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The potential benefits of repeated measure experiments for fish disease-challenge host-pathogen investigations

L. Malcolm Hall[a,**,2], Catherine Collins[a], Bertrand Collet[a,b,*,1]

[a] Marine Scotland Science, Aberdeen, AB11 9DB, UK
[b] Virologie et Immunologie Moléculaires, Institut National de la Recherche Agronomique (INRA), Université Paris-Saclay, Jouy-en-Josas, France

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ABSTRACT

The utility of molecular response data arising from *in-vivo* single and repeated measure fish disease-challenge experiments is compared. An *in-silico* 'experiment' involving the generation of two imaginary immune-molecule quantity response profiles over time for individual animals was carried out. Daily 'observed' molecule quantities were drawn from the 'known' individual response profiles to mimic the results of single and repeated measurement. The results indicate that repeated measure experiments are required to infer individual level response profiles, and that these experiments also provide more accurate summary statistics and data more suited to inferring the dependent ordering of the molecular response. Additionally repeated measure experiments utilise fewer animals than single measure experiments. These results are described alongside a discussion of experimental methodological issues pertinent to the adoption of aquatic animal repeated measure experimental designs. We conclude that investigators need to take particular care when making inferences from single measure experiments and that serious consideration should be given to using repeated measure experiments for *in-vivo* fish disease-challenge investigations.

1. Introduction

Immunologists are interested in the molecular processes of host-pathogen interactions in the expectation that a knowledge of these can be used to increase protection from disease (e.g. Ref. [1]). While some investigations can be carried out *in-vitro* (e.g. Ref. [2]) animal-based disease challenge experiments are required to relate molecular processes to clinical and sub-clinical outcomes (e.g. Ref. [3]). Such *in-vivo* experiments often involve challenging a group of fish with a pathogen or treatment and subsequently measuring RNA transcript or protein over one or more time-points using euthanased individuals. This involves sampling different fish at each experimental time-point (e.g. Ref. [4]). Measurements are often summarised at each time-point as averages and some estimate of variability (e.g. Ref. [4]) although more sophisticated statistical approaches may be used (e.g. Ref. [1]). Each individual is measured only once and such experimental designs, even though they involve multiple time-points, are hereafter referred to as 'single measure experiments'. An alternative approach is available. This involves measuring the quantities of RNA transcript or protein over several time-points by repeatedly sampling the same individuals [5]. Such experimental designs are hereafter referred to as 'repeated measure experiments'. This more intensive use of individual fish is associated with a higher precision [5] reducing experiment sizes in accordance with replacement, reduction and refinement (3R) principles [6] and legislation (e.g. Ref. [7]).

The authors have been evaluating the utility of repeated measure experiments to investigate host-pathogen interactions in salmonids (e.g. Refs. [8,9]). In this paper we further develop this evaluation by describing a computer-generated *in-silico* 'experiment' comparing the outcomes of single and repeated measure experiments. The results are discussed alongside a consideration of important methodological issues pertinent to the adoption of repeated measure experiments.

2. Approach

The *in-silico* experiment involves generating quantities of an imaginary immune-response molecule, hereafter referred to as the 'molecule', at 10 min intervals from the moment of 'challenge' with a

Abbreviations: au, arbitrary unit; PPCV, percentage packed cell volume

* Corresponding author. Marine Scotland Science, Aberdeen, AB11 9DB, UK.
** Corresponding author.
E-mail addresses: malcolm.hall@gov.scot (L.M. Hall), bertrand.collet@inra.fr (B. Collet).
[1] Corresponding author for aquarium and laboratory queries.
[2] Corresponding author for statistical queries.

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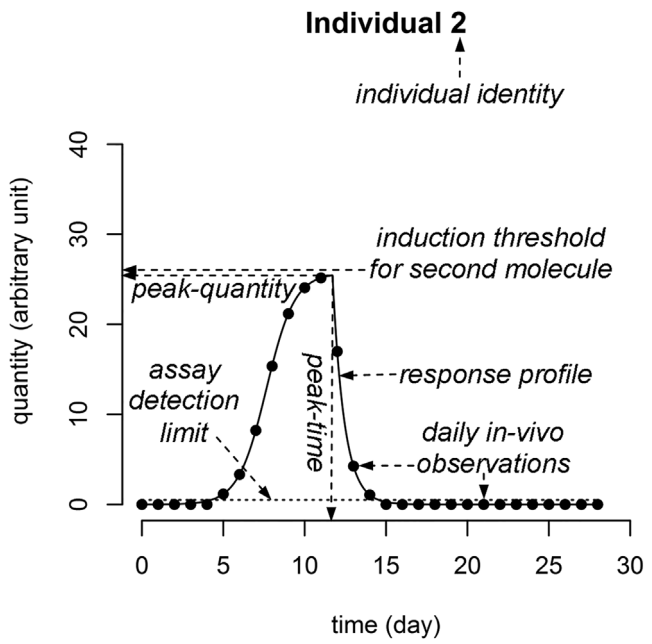

Fig. 1. An annotated example of the model output for an individual. The peak-quantity of the first molecule does not exceed the induction threshold of the second molecule which is therefore not expressed for this individual.

pathogen to 28 days inclusive for 30 imaginary individuals. This establishes a 'known' pattern of molecule quantities, hereafter referred to as a 'response profile' (Fig. 1), for each individual from which 'observations' mimicking more practicable *in-vivo* sampling intervals for both single and repeated measure experiments can be drawn. The mathematical model used to generate response profiles is not in itself important providing the patterns of molecule quantity over time are, at least under some circumstances, biologically plausible.

This experiment assumes that the molecule quantity before challenge is zero and would remain so without challenge. Challenge is assumed to instantaneously induce production of the molecule, albeit at low levels initially, which accumulates to a maximum quantity, hereafter referred to as 'peak-quantity', at a time-point referred to as 'peak-time' (Fig. 1). Molecule production is assumed to terminate at peak-time with quantities decaying to a very low level, effectively zero, before the end of the experiment at 28 days. Molecule accumulation to peak-time follows a three-parameter logistic equation and the subsequent decay a two-parameter convex negative exponential equation as detailed in the appendix. Peak-quantity is defined as a mean of 30.0 arbitrary units (au) occurring at a mean peak-time of 10.0 days post-challenge. Individual peak-quantities and peak-times are independent and vary around mean values with a coefficient of variation of 15%. Application of the accumulation and decay equations generates a response profile for each of the 30 individuals.

Production of a second imaginary immune-response molecule by an individual may be induced by the first molecule. Induction of this second molecule occurs during the accumulation of the first molecule at the instant it exceeds a value of 25.5 au, one standard deviation below the first molecule's mean peak-quantity. Production of the second molecule is only induced in an individual if the quantity of the first molecule exceeds this threshold.

Daily 'observed' molecule quantities are drawn from the individual response profiles at 24 h intervals to mimic an *in-vivo* repeated measure experiment. It is assumed that the laboratory assay, hereafter referred to as the 'assay', has a lower detection limit (Fig. 1) of 0.50 au (1.7% of the mean peak-quantity) with no other measurement error. The latter assumption is unrealistic within an experimental science context but facilitates this comparison of single and repeated measure experiments.

The 'observed' molecule quantities from the repeated measure observations are also used to mimic the summary statistics arising from a single measure experiment. A single measure experiment comparable to the repeated measure experiment would require 30 different individuals for each 24 h interval. While mimicking the single measure experiment with summary statistics based on the repeated measure data omits the sampling variation of different individuals at the different time-points it does facilitate the comparison of the two experimental designs at the average level.

The results of this investigation are described as four potential benefits of repeated measure experiments.

3. Results

There is a difference between the single and repeated measure experiment profiles (Fig. 2). The individual repeated measure experiment response profiles follow the logistic increase and exponential decrease specified by the equations. The averaged profile of the single measure experiment is smoother and more symmetrical. The profiles differ because they describe the individual and an averaged response respectively. It is concluded that repeated measure experiments are required to infer individual-level response profiles.

It may be that the intended research does not require individual response profiles and that descriptive summary statistics are satisfactory. These could include the time at which molecule accumulation commences, peak-quantity, peak-time, and the time at which molecule quantity decays to close to its original value. Peak-quantity and peak-time will generate the most distinct signals. 'Observed' estimates of peak-quantity and peak-time for the single and repeated measure experiments are compared to 'known' values in Table 1. The estimates from the repeated measure experiment are closer to the 'known' values than the single measure experiment. It is concluded that repeated measure experiments provide more accurate summary statistics for peak-quantity and peak-time than single measure experiments.

It may be that the intended research does not require descriptive summary statistics, and that information on the dependency of molecule responses on each other, hereafter referred to as 'dependent ordering', is satisfactory. The inference of dependent ordering requires both information on the sequential order of molecule production and evidence of an association between molecules. The repeated measure experiment is able to provide relevant data (Fig. 3) with 'observed' results, not surprisingly, corresponding to 'known' results (Table 2). Single measure experiments are also able to provide data relevant to the sequential order of molecule production (Fig. 3). However, data relating to an association between molecules is limited to observations of the co-occurrence of the molecules at single time-points (Table 3) because individuals are euthanased. The single measure experiment identified 52 co-occurrences of the two molecules from an equivalent total of 870 individuals. A random association between the two molecules would have generated, on average, 217.5 co-occurrences. There is therefore no evidence of a positive association of the two molecules. This is a because the first molecule is, at least in this experiment, often undetectable at the time-point that the second molecule becomes detectable. It is concluded that repeated measure experiments generate more appropriate data for inferring dependent ordering than single measure experiments.

It may be that the intended research does not require information on dependent ordering and that only information regarding the sequential order of molecule production is satisfactory. Single measure experiments are, as described in the previous paragraph, able to provide this information (Fig. 3). A sufficient number of challenged animals are required at each sampling time-point to provide a reasonable chance (at least 80%) of detecting the two molecules in a majority (more than 50%) of animals. A lower chance of detection (less than 80%) would generate considerable ambiguity as to whether unobserved molecules indicated a lack of

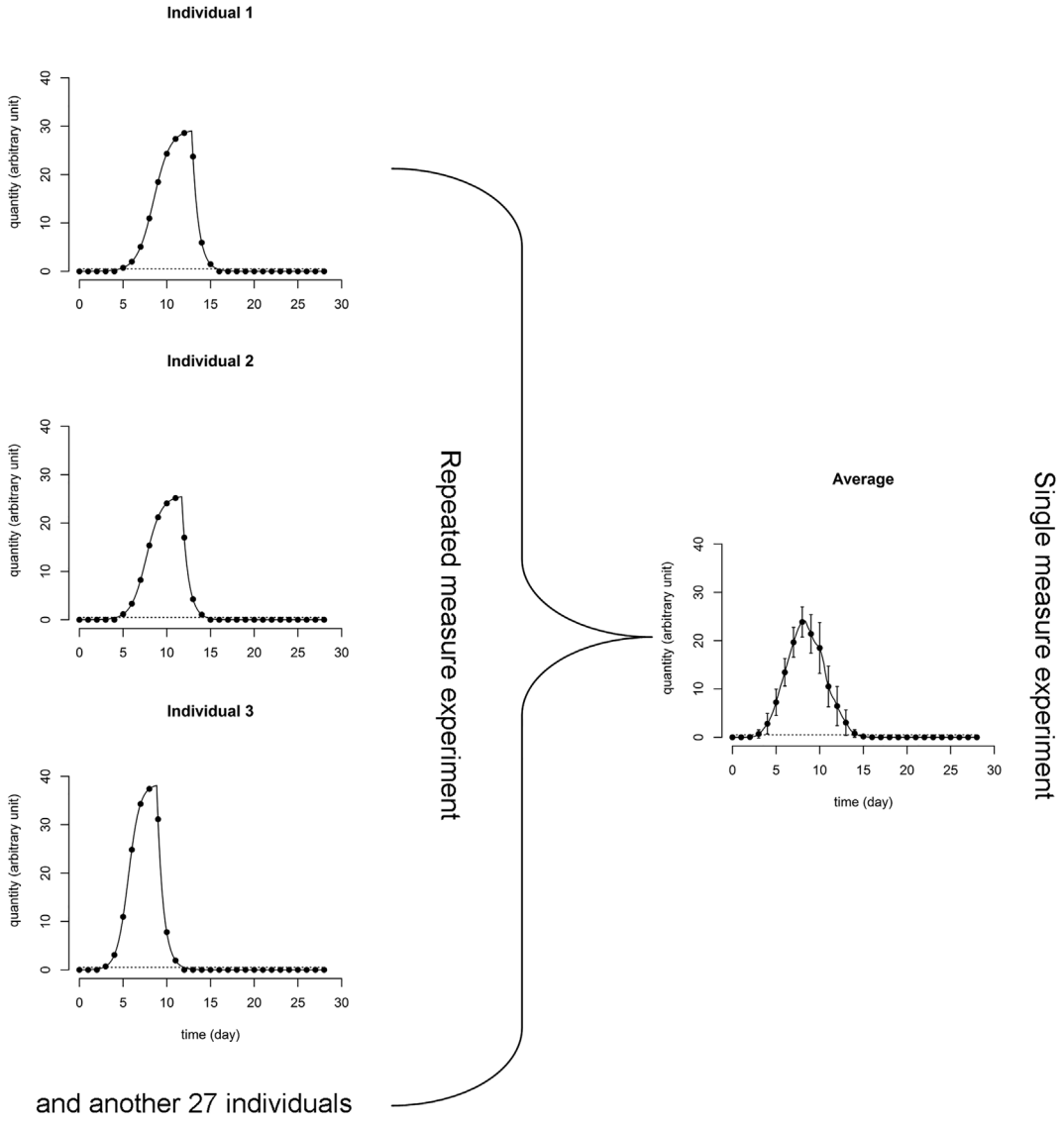

Fig. 2. Comparison of single and repeated measure experiment profiles. The continuous trace are 'known' molecule quantities. The solid circular points are 'observed' values at daily sampling time-points with those occurring below the horizontal dotted line being less than the assay detection limit. The vertical solid lines are 95% confidence intervals.

Table 1
Comparison of single and repeated measure experiment summary values.

	'Known' values[a]	'Observed' values	
		mean (range)	
	mean (range)	Repeated measure	Single measure
Peak-quantity	30.3 (20.8–40.0)	30.1 (20.8–39.9)	23.9 (1.2–37.4)[b]
Peak-time	9.7 (5.5–13.2)	9.3 (5–13)	8 (not available)

[a] For 30 individuals from peak-quantity $N(30,4.5)$, and peak-time $N(10,1.5)$.
[b] Range omits individual sampling variation between time-points.

expression in a majority of animals or were a consequence of using too few animals. Statistical power calculations, which assume that the two molecules are expressed independently, indicate that nine animals at each time-point are required; 261 animals for an experiment involving daily sampling on the challenge and 28 subsequent days. This is substantially larger than the 30 animals utilised for the repeated measure experiment. It is concluded that repeated measure experiments, even if only intended to infer the sequential order of molecule production, utilise fewer animals than single measure experiments.

4. Discussion

The results of this experiment indicate that repeated measure experiments are preferable to single measure experiments for both scientific and statistical reasons. While this experiment is contrived, in that it utilises an idealised mathematical model to generate imaginary data, the authors are not aware of evidence indicating that the response profiles are biologically implausible for at least some immunological response molecules. Additionally peak-quantities and peak-times can be altered to modify the response-profile and lead to the same conclusions (results not presented). There are, of course, other possible response profiles. These include, for example, the quantitative polymerase chain-reaction detection of *mx* gene transcripts in Atlantic salmon smolts before challenge which, following challenge, persist at an increased quantity after peak-quantity [8]. Modelling this type of response-profile would require an alternative mathematical model, although a partial solution assuming a non-zero but equal pre-challenge and final molecule quantity is available by simply adding a constant value to all the model outputs as a final step. Likewise response profiles defined by different accumulation and decay equations, some of which describe even more transient expression, can be envisaged. Given the paucity of published *in-vivo* experimentally derived response profiles for

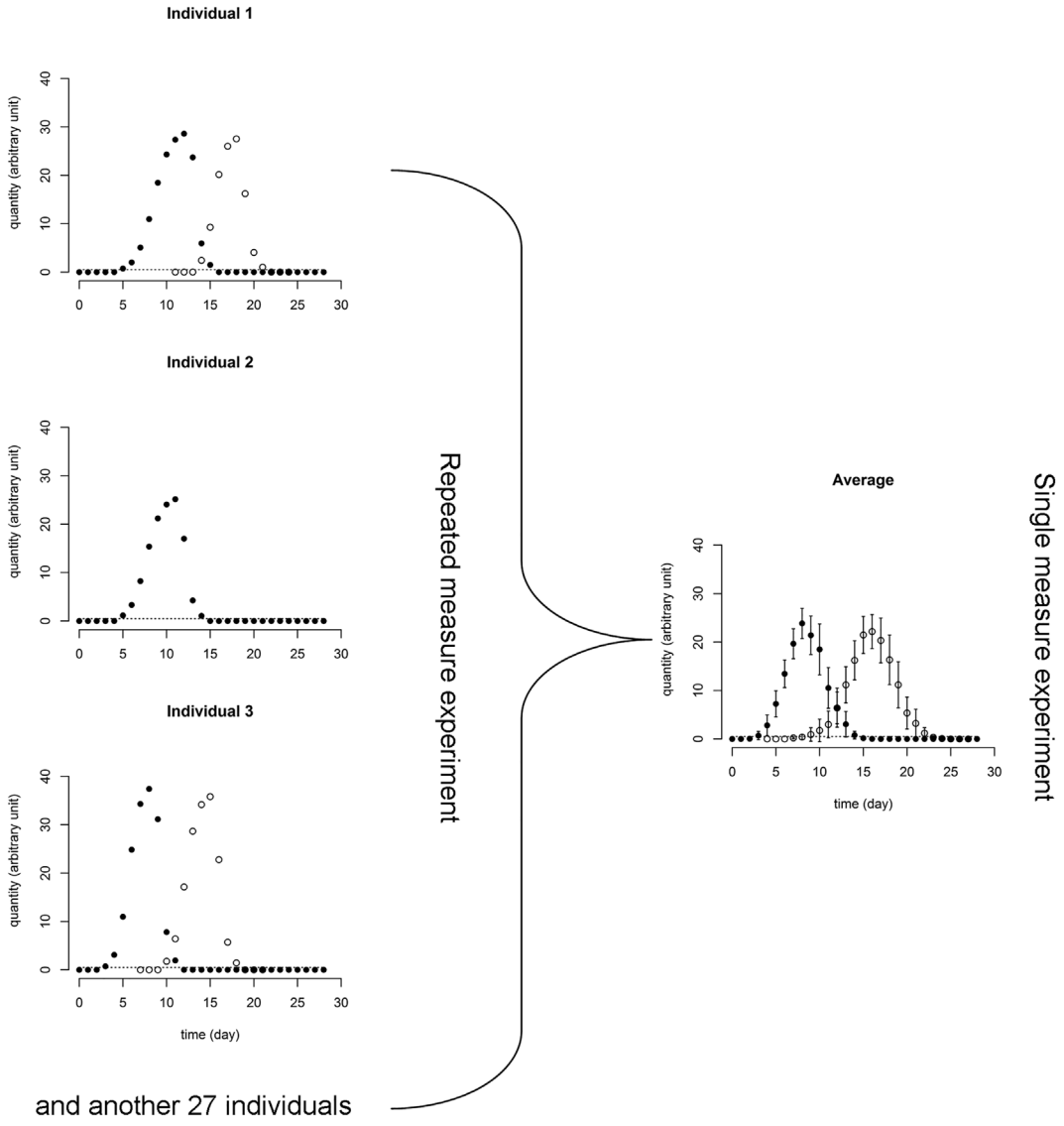

Fig. 3. Comparison of single and repeated measure experiment 'observed' values for sequentially induced molecules. Solid and open circular points are the 'observed' values at daily sampling time-points for the first and second molecules respectively. The second molecule is only induced when the first molecule exceeds 25.5 au. 'Observed' values occurring below the horizontal dotted line are less than the assay detection limit. The vertical solid lines are 95% confidence intervals.

Table 2
Correspondence between 'known' and 'observed' occurrence of second molecule from repeated measure experiment.

		'Observed' occurrence	
		Present	Absent
'Known' occurrence[a]	Present	23[b]	0
	Absent	0	7

[a] 'known' because the first molecule exceeded the induction threshold for second molecule.
[b] From 30 individuals measured daily on days 0–28 inclusive (30 animals in total).

Table 3
Correspondence between 'observed' occurrence of first and second molecule from single measure experiment.

		Second molecule	
		Present[a]	Absent
First molecule	Present[a]	52[b]	230
	Absent	172	416

[a] Molecule is defined as present if exceeding 0.50 au.
[b] From 30 individuals euthanased daily on days 0–28 inclusive (870 animals in total).

individuals, the equations utilised for this experiment can be regarded as appropriate for comparative purposes. The demonstration, therefore, that repeated measure experiments are capable of generating better quality data utilising fewer animals than single measure experiments for at least some possible response scenarios warrants, at the very least, serious consideration.

The results have focussed on the potential benefits of repeated measure experiments. It would be irresponsible, however, not to describe some of the practical constraints to this approach. First, repeated measure experiments only allows for the sampling and examination of specific internal organs at the experiment's termination. This limits investigations into the association between molecular responses and the development and resolution of pathology. The identification of fish-based plasmatic biomarkers to inform on tissue damage is progressing however (e.g. Ref. [10]; [11]). While the characterisation of such biomarkers for individual inference requires further development, they provide a potential tool to infer tissue damage and recovery

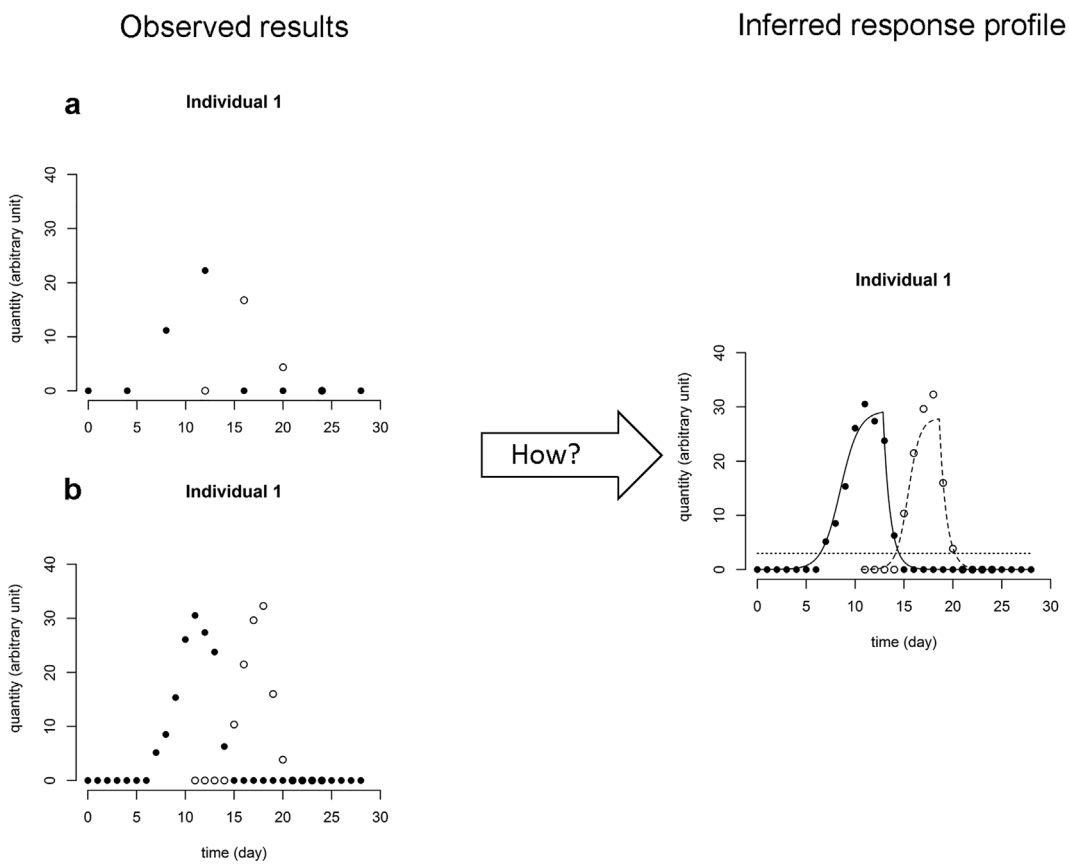

Fig. 4. Inferring response profiles from experimental observations given different measurement intervals. Solid and open circular points are the 'observed' values for the first and second molecule respectively at either four-day (a) or one-day (b) measurement intervals. The continuous and dashed traces are the 'known' response profiles to be inferred for the first and second molecule respectively. The assay error is assumed to be 15% of the mean peak quantity. The assay detection limit, shown by the dotted horizontal line, is assumed to be 3.0 au with a value of '0' indicating either the absence of the molecule or its presence below the limit.

during repeated measure experiments. We posit that the requirement to destructively infer pathology will become less necessary as such biomarkers are further characterised and that this will facilitate repeated measure experiments.

Statistical design and analysis represents a second practical constraint with repeated measure experiments requiring an expert statistical input [5]. It is the opinion of the authors, however, that the types of repeated measure experiment described in this paper require more than a contribution of existing statistical expertise. Rather optimal fish-based repeated measure experimental designs investigating molecular host-pathogen or treatment interactions are yet to be established, and the reliability of the statistical methods used to analyse such data require validation. For example, is it possible to infer molecule response profiles utilising a four-day measurement interval with assay error? Data from a one-day measurement interval appears to be very much more preferable (Fig. 4). Even then, however, the statistical methods required to reliably infer ordered dependence, summary statistics or, ideally, response profiles are, at best, not validated within this context. In the authors' opinion some of these uncertainties can be overcome through quantitative *in-silico* investigations similar to those described in this paper.

Measurement interval represents a third practical constraint to repeated measure experiments. The mention of a four-day measurement interval in the previous paragraph was intentional because it represents the authors' experimentally validated minimum using blood from salmonids [8,9]. The sampling of blood volumes just above 10% at intervals of less than four days was associated with an unstable blood percentage packed cell volume (PPCV), an increased proportion of immature blood cells, and an increased expression of molecular stress markers [9]. In contrast PPCV and similar stress markers remained stable for uninfected animals during an experiment which sampled less than 10% of blood at intervals of four days [8]. The authors' continuing experience indicate that the volumes of blood required for assay can be reduced, thereby providing a potential opportunity to reduce the measurement interval in relation to obtaining usable amounts of material. Alternatively, it is possible to sample mucus non-lethally, with the impact of ecto-parasitic diseases on salmonid aquaculture [12] stimulating work on the immune involvement of this material in its own right (e.g. Refs. [13,14]). In the authors view, however, further experimentation is required to evaluate to what extent this material is representative of the induced mucosal or systemic immune response.

The quantity of material sampled is not, however, the only constraint on measurement interval with the processes involved in sampling also potentially affecting results. While the use of in-tank anaesthesia [8] should eliminate stress responses associated with chasing, netting and handling prior to anaesthetic, as well as reducing damage to skin and mucus barriers, the anaesthetic itself may affect results. For example the use of eugenol (4-allyl-2-methoxyphenol), which given its physical properties is likely to coat gill epithelia [15] and when repeatedly administered as clove oil has been associated with mild gill necrosis [16], is most likely inappropriate as an anaesthetic for repeated measure experiments utilising gill mucus. And while anaesthetisation has, for example, been shown to decrease the cortisol release associated with at least some forms of stress (e.g. Refs. [17,18]), there is evidence of some increase in cortisol associated with the process of anesthetisation itself [19]. Methodological improvements such as utilising pre-sedation and combinations of anaesthetics (for a review see Ref. [20]), approaches to reducing hypoxia (discussed by Ref. [21]), and the potential use of cannulation (e.g. Ref. [22]) may further reduce the stress associated with anesthetisation. Additionally, the direct effect

of anaesthesia on the pathogen cannot be discounted (e.g. Refs. [23,24]). Differences in response arising from repeated anaesthesia on either the animal or pathogen can be evaluated by comparing the results at the averaged level from parallel single and repeated measures experiments.

The *in-silico* results reported in this paper indicate that repeated measure experiments are intrinsically preferable to single measure experiments for both scientific and statistical reasons. The above practical constraints indicate, however, that further experimental and statistical research is required before the potential benefits of repeated measure experiments can be fully realised. While it would be rash, given the practical constraints described in this paper, to recommend that repeated measure experiments be adopted as 'the' default design for fish disease-challenge host-pathogen or treatment investigations, serious consideration should be given to their use. In the meantime investigators should also take particular care when making inferences from single measure experiments since such results are averaged and are not necessarily an accurate representation of individual responses. Ultimately, while repeated measure fish disease-challenge experiments cannot replace the use of animals, their adoption would at the very least reduce the number of animals used with any research undertaken to realise their full potential improving animal welfare.

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Appendix

The logistic accumulation equation to peak-quantity is:

$$y_{it} = \frac{a_i}{1 + (576 b_i \times e^{-144ct/141 b_i})}$$

where y_{it} = 'known' molecule quantity for individual i (1–30) at time t post-induction (0 to b_i days); a_i = peak-quantity for individual i randomly drawn from $N(30.0,4.5)$; b_i = peak-time in days post-induction for individual i randomly drawn from $N(10.0,1.5)$; c = maximum value of b across i individuals; t = post-induction time in days (0 to b_i). Induction times are from the moment of challenge for the first molecule and the moment the first molecule exceeds a molecule quantity of 25.5 au for the second molecule. Note that the equation utilises non-standard parameterisation to generate similar biologically plausible models across the range of a_i and b_i.

The exponential decay equation from peak-quantity is:

$$y_{it} = y_{ib_i} \times 2^{-(t-b_i)/d}$$

where additionally y_{ib_i} = 'known' molecule quantity for individual i (1–30) at b_i; d = molecule half-life (0.5 day); t = post-induction time in days (b_i to ≤ 28). Note that y_{ib_i} is slightly less than a_i because the logistic equation defines the latter as asymptotic.

It is necessary to add the induction time of the second molecule to generate the response profile of the second molecule relative to the start of the experiment. The equations are implemented within the R statistical environment version 3.3.3 [25].

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