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Errors in experimental design and statistical analysis of aquaculture diet evaluation studies induced by filtration systems

Michael F. Tlusty

Edgerton Research Laboratory, New England Aquarium, Boston Massachusetts, USA.
E-mail: M. F. Tlusty, mtlusty@neaq.org

Abstract. While single factor diet studies appear to be of simple design, the space constraints in aquaculture caused by the need to filter the aqueous media often create constraints on the implementation of the experimental design. A number of papers have been published in which the experimental design and subsequent analysis is incorrect. This paper reviews the principles of experimental design as it related to single factor diet evaluation studies in aquaculture. By adhering to simple design principles to ensure that experimental units are independent, the data generated by future experiments will be of high quality and results more robust.
Key Words: diet evaluation, blocked-design, experimental design, statistics.

Összefoglalás: Az étrendet vizsgaló kutatások, melyek egyetlet tenyező behatását vizsgálják egyszeru vazlatra alapozodnak. A halgazdálkodásban előálló térgátlások, melyeg a felhasznalandó víz szürésének következményei, jelentösen megfékezik a kutatási elgondolás végrehajtását. Számos tudományos dolgozatok kimutatták, hogy a kutatási elgondolas kivitelezése illetve az ezt követő vizsgálatok sok esetben helytelenek. A jelelnlevő dolgozt, áttekinti az alapveto kutatasi elgondolásokat, melyek a halgazdálkodásban végrehajtott étrendi kutatásokat celozzak, egyetlen befolyasoló tenyező korulmenyei közt. Betartva az egyszeru elgondolás alapelveit, illetve a kutatási csoportok egyedüléllóságát, sikeres és nagy ertekü adatok gyüjtését bisztosíthatjuk.
Kulcsszavak: etrend ertekeles, gatlott-elgondolas, kutatasi elgondolas, statisztika.

Rezumat. În timp ce studiile de alimentaţie care implică un singur factor par să aibă un design foarte simplu, necesităţile de spaţiu pentru filtrarea mediilor apoase în acvacultură creeaza anumite constrângeri în implementarea designului experimental. Un număr de lucrări ştiinţifice au fost publicate, lucrări în care designul experimental şi analiza statistică sunt incorect alese. Această lucrare dezbate şi revizuieşte principiile designului experimental asemănător studiului ce implică evaluarea unui singur factor nutriţional în acvacultură. Aderând la principiile designului simplu pentru a ne asigura că unităţile experimentale sunt independente, datele generate de experimentele viitoare vor avea o calitate mai bună, iar rezultatele vor fi mai solide.
Cuvinte cheie: evaluare nutriţională corectă, dietă, design experimental, statistică experimentală.

Introduction. The design of experiments to examine how diet affects animal performance is fairly straight forward. In its basic form, a group of animals are divided into groups equal to the number of diets, diet components, or level of nutrient that need to be assessed. The animals are then fed the experimental formulations for some pre-determined amount of time. While this is a typically a straightforward exercise, within aquaculture, the nature of the specialized aqueous environment can present challenges that may compromise the robustness of any experimental design.

Because of the need to filter the water in which aquatic animals live, the filter system may constrain the implementation of an experimental design. The constraints come primarily via the filtration system. In a number of studies, it is unusual that the experimental tanks are either filtered individually, or are all connected to the same filtration system. Typically, the experimental system has a number of filters processing the water for a subset of tanks. A second variant on the design constraint theme is that the tanks are divided into multiple water bodies (e.g., rearing trays or header tanks) partitioned off of a single larger filter system. In either case, the filtration system needs to be accounted for in the statistical analysis, and by not doing so, the statistical significance of the results can be overstated and in a worst-case-scenarios, erroneous.

Recently, a number of studies have been published that assess the role of adding salt to diets of juvenile fish in order to improve growth and survival (Table 1). Here, the experimental designs of these studies are reviewed in an attempt to discern how improper set up and analysis with respect to filtration system and the spread of dietary treatments across the filtration systems may influence the results of these studies. Ideas are also presented for how slight adjustments to these studies could improve their statistical rigor.

Design Mistakes. A number of researchers have inappropriately designed and analyzed the salt addition diet studies, and while it is not the intention to single out any one study, the example to be used in this paper will be the recent paper published in this journal by Arockiaraj & Appelbaum (2010). This study examined the effect of dietary salt addition on the growth and performance of Asian seabass *Lates calcarifer* (Bloch, 1790). Within their design, they fed the fish five different salt diets. Each diet was replicated three times and a total of 15 tanks were used in this study. Each of the replicated tanks were all attached to a singular filter unit (n = 5). This design is demonstrated in Fig. 1 (design 1) with five groups of three tanks.

Table 1

A non-exhaustive list of studies that have examined how dietary salt addition effects fish performance. The number of diet treatments replicates, and total number of tanks are provided. The filtration units indicate the number of independent filters used for the experiment, and in all cases where this number > 1, the data were incorrectly analyzed

Reference	# filter units	# diet treatments	replicates	Total tanks	Analysis
Appelbaum & Arockiaraj 2009	4	4	3	12	One-way ANOVA
Arockiaraj & Appelbaum 2010	5	5	3	15	One-way ANOVA
Cnaani et al 2009	6	5	7	35	One-way ANOVA
Fontainhas-Fernandes et al 2000	1	2	3	6	Multifactoral*
Gatlin et al 1992	1	3	3	9	One-way ANOVA
Harpaz et al 2005	2	4	3	12	Two-Way ANOVA**
Shiau & Liu 2004	1	7/8***	3	21/24	One-way ANOVA

* also sex and water salinity treatments.

** this experiment used a nested design (incorrectly analyzed as a factoral ANOVA) with water salinity being the second factor, but no accounting for the filtration units.

*** two different experiments were conducted

The diets were fed to the fish in the tank, and the assumption of the authors is that the tank is the experimental unit. However, the error with this experimental design is that the diet treatments now lack independence. While the diet is being fed to the individual tank (the intended experimental unit), the tanks cannot be differentiated as independent replicates because they all are linked into the same filtration system. This is a case of pseudoreplication (Hurlbert 1984) where the number of observations is increased by making multiple observations on the same functional unit. In this study, Arockiaraj & Appelbaum (2010) observed statistically significant differences between the diet treatments. However, because each diet is on a different filtration system, it cannot be said with certainty if the treatment differences are a result of the filtration system or the diet. What if salinity within a filtration system shifted as a result of leaching from the diet? Perhaps the bacteria colonizing the biofilter are different as a result of slight but immeasurable water quality differences. There are too many unanswered possibilities for alternate explanations as to why treatment differences were observed. Unfortunately, with this design, it is impossible to determine with accuracy if the observed result is because of the experimental manipulation or an artifact of the experimental set-up.

Correct Experimental Designs. There are number of possibilities for modifying the design of this experiment to correct the pseudoreplication problem and sampling deficiency and to be able to make a meaningful statistical analysis. The first is to connect all tanks to a single large filtration system so they are all functionally in the same water body. This would provide 14 (n-1) replicated independent experimental units (the tanks) and would allow for statistical testing with a one way ANOVA with the F value calculated with 4 treatments (5-1) and 10 error degrees of freedom (Fig. 1, design 2). This does raise the question of the entire experiment being conducted on a single filtration unit, but this is the bane of laboratory experiments. Any experiment is theoretically only valid for the conditions within that laboratory. Thus, theories on diet performance, such as how salt will impact performance, need to be examined first in the laboratory, and then confirmed in a second laboratory, and the field tested. To overcome the issue of a single filtration system running the entire experiment, a slightly different solution would be to utilize independent filters on each of the 15 tanks. While this is functionally a different system design, statistically it would be treated similarly, and the statistical significance of an ANOVA would be examined with $F_{4,10}$.

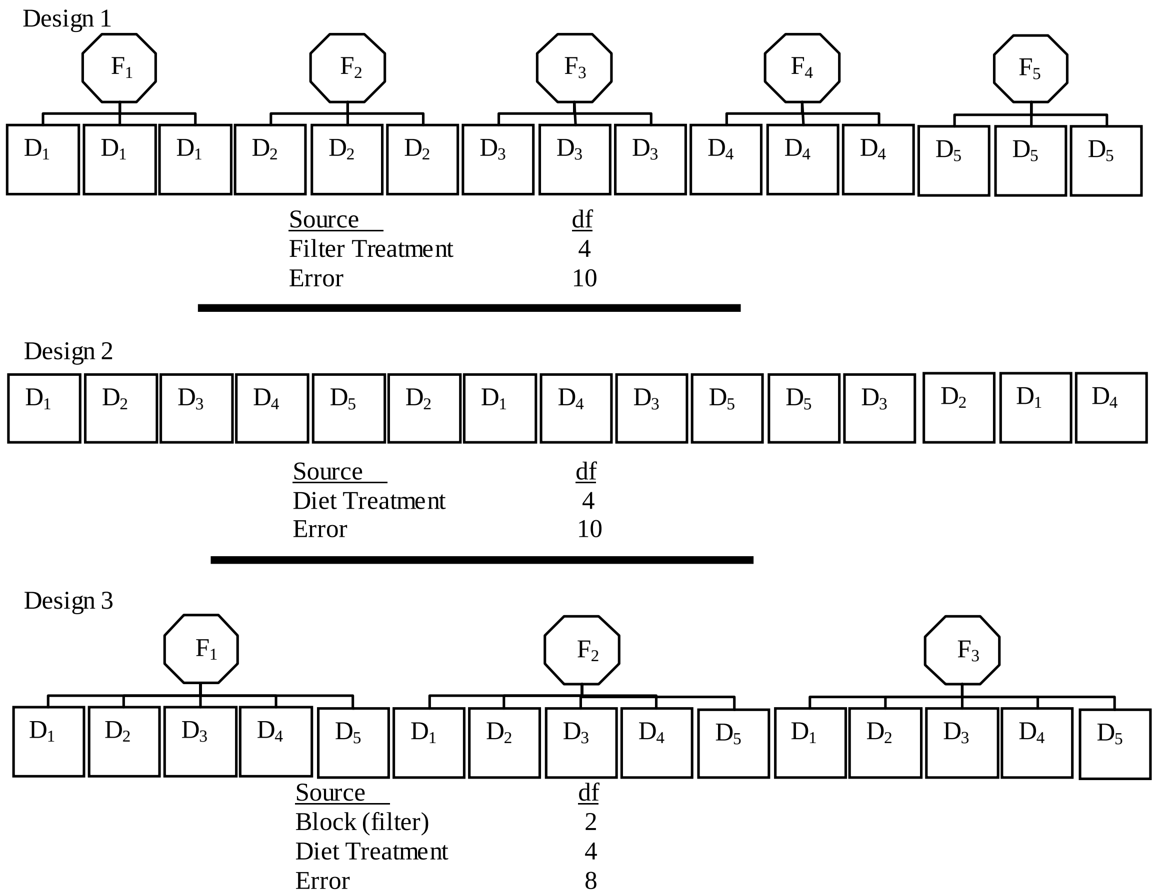

Figure 1. Different single diet experimental designs based on various tank-filtration system designs. Filtration unit is designated by F, diet by D with numbers referring to separate systems and treatments respectively. Design 1 is the incorrect design as discussed within, while 2 and 3 are distinct alternatives. The ANOVA listed below each design is how the experiment was (Design 1), or should be analyzed (Designs 2 and 3).

If these first two options are not available, and it is necessary to utilize an intermediate sized filter that cannot adequately filter all the tanks, then as opposed to placing a single treatment on each individual filter system, it is best to randomly distribute the experiment units (the diet-treatment-tanks) uniformly across each filtration system in a randomized complete block design. In this case, diagrammed within Fig 1 (design 3), the experimental units (diet-treatment-tanks) are connected to the separate filtration systems (blocks) with an equal number of experimental units within each of the blocks. In the case described within Figure 1, it would be most judicious to utilize 3 filtration systems. There are still 14 (n-1) independent experimental units, however, the statistical significance of an ANOVA would be examined with $F_{4,8}$. The error degrees freedom is reduced in this case since error has to be attributed to the blocking factor. In this case, the total degrees of freedom (14) is reduced because of the treatment d.f. (4) and the block d.f. (2) for an error d.f. of 8. While it would seem that the block design will be of lower statistical power because of the reduced error degrees of freedom, this may not be the case. In a derived data set equivalent to design 3 in Figure 1, conducting the analysis as a one-way ANOVA does not yield a significant diet treatment effect (Table 2). However, accounting for the three filters and analyzing the data as a blocked design results in a significant diet treatment effect (Table 2). While this result may seem counterintuitive (cannot discern treatment differences with more df in the error term), it is a result of partitioning error to the filters, and thus reducing the overall mean square error (MSE) term in the denominator that results in a significant diet treatment effect within the blocked analysis. The MSE of the error term when the data are analyzed as a one-way ANOVA was 0.098, where for the blocked analysis, it was 0.062. Therefore, accounting for the filter by utilizing a blocked analysis will create the more robust analysis.

Table 2

A derived data set examining treatment effects of five diets, each replicated three times, and distributed over three filtration systems in a randomized complete block design (design 3 in Fig. 1). The data were derived as random values with diets 4 and 5 being a multiplied by a factor of two. The analyses below are if the data were assessed as a single factor experiment with a one-way ANOVA, or if they were analyzed as a blocked design. Statistical similarity (Tukey's HSD) was signified by like superscripts following the mean value. Within the ANOVA tables, SSE and MSE refer to sum of squares error and mean square errors respectively

			Diet		
Filter	**D_1**	**D_2**	**D_3**	**D_4**	**D_5**
F_1	0.618	0.380	0.505	0.570	0.957
F_2	0.762	0.927	0.831	1.390	1.247
F_3	0.147	0.815	0.961	1.222	1.441
Mean	0.509^{a}	$0.707^{a,b}$	$0.766^{a,b}$	$1.061^{a,b}$	1.215^{b}
95% C.I.	0.364	0.327	0.266	0.490	0.275

Source	***df***	***SSE***	***MSE***	***F***	***P***
one-way ANOVA					
Diet	4	0.964	0.241	2.463	0.113
Error	10	0.978	0.098	0.113	
Total	14	1.942			
Blocked Analysis					
Filter	2	0.485	0.242	3.926	0.065
Diet	4	0.964	0.241	3.904	0.048
Error	8	0.494	0.062	0.062	
Total	14	1.942			

Discussion. While the accounting for filtration system constraints may appear to be a minor point, it can have significant ramifications. This design will impede progress toward the true assessment of the performance curves of fish fed different levels of dietary salt inclusion. From the work conducted by the number of authors (Table 1), it appears that there is an intermediate level of salt that optimizes performance. Determining the true optimal value will be difficult because the single experimental unit will lead to a less robust estimate of the performance value. Overall, it will take more effort to determine the optimal level of dietary salt to include in diets, compared to if the experiment was appropriately designed and analyzed.

Unfortunately, neither of these solutions offered in Figure 1 (designs 2 and 3) can be applied to the extant problematic datasets as described (sensu Fig. 1a). The separation of diet treatment groups within singular filtration systems does not allow for statistical analysis because ultimately there is a sample size of 1 (filtration system) per diet treatment. In addition, it must be remembered that not all tanks or filtration units are equal regardless of the effort directed at making them similar. In diet work conducted by this author (Myers & Tlusty 2009), system constraints necessitated a design where six experimental groups were divided into two separate trays (an upper and a lower) with three treatments per tray, all connected to a single filtration system. The analysis of these data as an incomplete blocked design demonstrated that there was a significant difference between the two trays, which complicated the analysis. It would have been a more prudent experimental design to place each diet treatment into each tray in a randomized complete blocked design.

The experimental designs discussed within this review are fairly simple as dictated by the single diet factor studies. More complex designs may be warranted if additional factors are included within studies. If multiple factors are being assessed (e.g. diet and sex), it is the researcher's prerogative to design the experiment according to designs presented in an established manual. The FAO (Nokoe 1992), provides a publically available document which describes various experimental designs, and the statistical analysis appropriate for the design. There are also a variety of online resources that can help differentiate between blocked, nested, and split-plot among other designs.

In the case of the salt inclusion work, the number of studies reporting positive results leads to the summary conclusion that including salt does improve the performance of many species. One day, a meta-analysis (Arnqvist & Wooster 1995) may be possible, but this is currently difficult because of the wide variety of species, methods, designs, and salt inclusion levels examined. The goal of this paper is to clear confusion about how to best design these experiments and to move forward, generating robust data that can be effectively analyzed and efficiently implemented into best feeding practices.

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Author:
Michael F. Tlusty, Edgerton Research Department, New England Aquarium, 1 Central Wharf, Boston Massachusetts, 02110, USA; e-mail: mtlusty@neaq

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