PRINCIPLES OF EXPERIMENTAL DESIGN

Chapter 4
INTRODUCTION

- An experimental study is a scientific test that investigates the relationship between an outcome and one or more conditions manipulated by the researcher.

- Before considering the appropriate experimental design, it is important to be clear about the **aims of any experiment**, which are usually associated with one or more scientific questions or hypotheses to be tested.

- A thorough definition of the **objectives of the design** is required to make easy to assess whether the chosen treatments are sufficient the assess these aims.

- The term **treatment** will be understood as the set of different experimental conditions to be tested.

- Asides from identifying the experimental treatments, **experimental units** must be chosen. Experimental Units (EU) are “the smallest division of the experimental material such that any two units may receive different treatments in the actual experiment”.

Examples of EU are:

a) An area of land on a farm (e.g. a field plot)
b) Individual soil samples taken from a field
c) Pots, each containing three plants
d) Different leaves from an individual plant
e) Sub-samples of RNA extracted from one plant
f) A batch of ten insects in a Petri dish.
INTRODUCTION

- EU’s should be chosen to be *reasonably homogeneous* so that treatments are compared on a fair basis.
- Sometimes an intrinsic structure is found in them, introducing heterogeneity between groups of more homogeneous units, and this can be *considered* in the experiment.
- EU are chosen according to the frame of reference for the experiment.
- **TREATMENTS** are chosen to enable the experimental hypotheses to be tested.
INTRODUCTION

- **Observational or measurement units** does not necessarily have to be the EU. Measurement units are units on which the individual observations are recorded.

- Underlying unit-to-unit variation in biological/physical experimentation is always present.

- Natural variation may be inflated by **measurement variation** (also known as measurement error). This combined background variation is a potential cause of both bias and uncertainty in experimental results.
Experimental design and statistical analysis aims to:

1) Distinguish
2) Quantify and
3) Compare the effect of treatments (signal) and background variation (noise).

- Large \textit{signal:noise} ratio provides \textit{indication} that significant differences between treatments are present.
- Small \textit{signal:noise} ratio indicates that apparent treatment variations can be explained mostly by background variation.
INTRODUCTION

- **Good** experimental design matches EU and treatments such that treatment differences are estimated:
  1) Without bias or without systematic over/under estimation.
  2) As precisely as possible, by minimizing uncertainty in the results.

- **Experimental bias** is avoided by *random allocation* of treatments to EU (i.e. *randomization*).

- **Precision** is attained by proper *replication* and *blocking*. These are the three basic principles of good experimental design.
INTRODUCTION

Replication:

The process of applying each treatment to more than one EU, so that the number of independent EU to which the treatment is applied to is the number of replicates.

Randomization

Randomly allocating treatments to EU, to ensure fair assessment of the treatments. It provides insurance against potential unknown biases.

Blocking

The process of identifying or building groups of EU which are expected to have similar responses in the absence of any treatment effects. Blocks are subsets of experimental material within which EU are expected to be homogeneous, with more heterogeneity allowed between EU in different blocks.
“Once the aims of the experiment have been defined, and the appropriate treatments and experimental units chosen, the three basic principles of experimental design: randomization, replication and blocking, give confidence that any treatment differences observed are real and not due to some chance combination of circumstances.”
REPLICATION

Process of applying each treatment to more than one experimental unit

Uses of replication:

1) Repeating each treatment on several EU attains a more reliable estimate of the effect of each treatment.

2) Replicated observations provide an estimate of background variation between units, which can be used to assess the importance of treatment differences.

\[ n_j \] (number of times a treatment \( j \) is applied)

Simplest case (equal replication): \( n_1 = n_2 = n_3 = \ldots = n_t = n \)
REPLICATION

- **Pseudo-replication:** when observational units within an EU can be mistaken for replications.
- It leads to an incorrect estimate of between unit variability.

*True replication vs pseudo-replication:*

Sub-samples are pseudo-replicates

Guideline:
In order to count as real replication, units with the same treatments should not be more closely associated than units with different treatments.
MICE ON CAGES

EXAMPLE

- 6 cages, 5 mice / cage
- 2 treatments, each applied to 3 cages

- What is the experimental unit – a mice or a cage?

  A cage – because treatments are applied to whole cages

- What is the true replication?

  \[ n = 3 \]

  \[ m = 15 \text{ mice / cage (pseudo-replication)} \]
Technical vs Biological replication

**Technical replication** involves the repeated measurement of the *same* sample. It is important in controlling for errors in measurement or technology. This type of replication is always considered pseudo-replication.

**Biological Replication** takes place when measurements are taken from several independent biological subjects rather than from a single individual. It is important in the statistical inference of populations.
**REPLICATION**

**EXAMPLES**

a) 12 pots, each containing 4 plants at the 3-leaf stage, with six treatments, each applied to two of the pots selected at random.

![Diagram of pots and treatments](image)

b) 12 plots, with two treatments, each applied to six of the plots selected at random. One soil sample was taken per plot, then the soil samples from the six replicate plots for each treatment were bulked together and mixed thoroughly and six sub-samples taken and measured.

![Diagram of plots and treatments](image)
REPLICATION

EXAMPLES

c) Two controlled environment cabinets, one at 10°C and one at 20°C, each containing eight seed trays, with two different watering regimes each applied to four trays within each cabinet chosen at random.

\[
\begin{array}{cc}
10°C & 20°C \\
B & A \\
B & B \\
A & A \\
A & B \\
\end{array}
\]

d) Twelve field plots, 2 treatments each randomly applied to 6 plots, height of 25 plants per plot measured each week for 4 weeks.
Randomization ensures *fair* allocation of treatments to units, guarding the design against *bias* and coping with the *natural variation* between EUs.

To obtain a proper randomization for a given design, a *method* is required for assigning treatments to experimental units at random.
RANDOMIZATION

• Randomization ensures that any *bias of the experimenter* is avoided and that any *unknown* differences between the units are unlikely to consistently favor particular treatments.

• Randomization in its simplest form is such that each permutation of the set of treatments has equal probabilities, so that every EU has the same chance of receiving any treatment.

• It usually is a good practice to conduct the experimental measurements without knowledge, as far as possible, of treatment allocation (e.g. double-blind test).

“Do a double-blind test. Give the new drug to rich patients and a placebo to the poor. No sense getting their hopes up. They couldn’t afford it even if it works.”
RANDOMIZATION

- EUs should always be chosen as homogeneous as possible, but still be representative of the population.
- Randomization it does not guarantee that any individual randomization will be unbiased.

**EXAMPLE**

A A A A A A A A B B B B B B B B

(which occurs with probability 1 in 12870)

If this occurs:

1) You need to implement some blocking!
2) Consult a statistician to discuss what to do!
The efficacy of a new pesticide is to be tested in the field using fifteen 5 m × 10 m plots arranged in a 3 × 5 array. Initially five plots will be sprayed with the pesticide and ten will be untreated (controls) for comparison.

Method 1

- From a deck of cards we take 15 cards, 5 red to represent pesticide treatment (P) and 10 black representing the control treatment (C).
- The cards are shuffled (randomization).
- The cards are dealt in the order they come up once shuffled, to allocate the treatment to plots, obtaining for example the following randomization:

```
1 P  2 P  3 C  4 P  5 C
6 C  7 C  8 P  9 C 10 P
11 C 12 C 13 C 14 C 15 C
```

Is Method 1 valid to randomize the treatments assigned to the EU?
Method 2

- Toss a coin to choose the treatment to be applied, with heads corresponding to P and tails to C.
- The coin is tossed for each of the plots and P is assigned if heads comes up (provided that the number of plots assigned to P up to that moment are less than 5) or C if tails comes up (subject to a max of 10).

<table>
<thead>
<tr>
<th>PLOT</th>
<th>Treat</th>
<th>PLOT</th>
<th>Treat</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C</td>
<td>9</td>
<td>P</td>
</tr>
<tr>
<td>2</td>
<td>P</td>
<td>10</td>
<td>C</td>
</tr>
<tr>
<td>3</td>
<td>C</td>
<td>11</td>
<td>C</td>
</tr>
<tr>
<td>4</td>
<td>P</td>
<td>12</td>
<td>C</td>
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<tr>
<td>5</td>
<td>P</td>
<td>13</td>
<td>C</td>
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<tr>
<td>6</td>
<td>P</td>
<td>14</td>
<td>C</td>
</tr>
<tr>
<td>7</td>
<td>C</td>
<td>15</td>
<td>C</td>
</tr>
<tr>
<td>8</td>
<td>C</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Is Method 2 a valid randomization of the treatments assigned to the EU?
RANDOMIZATION

Methods for randomly allocating treatments to units

- flip a coin?
- throw a die?
- pick numbers from a hat?
- select cards from a pack?
- use random number tables?
- run computer packages? ......

- Simplest experimental design: CRD

- Each treatment is equally likely to be applied to any experimental unit within the experiment.

It is allocation of treatments to units not units to treatments
BLOCKING

• It is not always possible to have reasonably homogeneous EUs (as they are intrinsically heterogeneous)

• However, it is possible to identify **groups** of EUs, such that within such groups EUs are *reasonably* homogeneous, but heterogeneous across different groups.

• Information on the causes of heterogeneity is used to define blocks.

• Blocking has to be done carefully: blocking where there is no necessary reduces the precision of treatment comparisons.
**BLOCKING**

**Completely randomized design:**
- All treatments have equal chance of being on top shelf, so unbiased.
- Some treatments may get lucky and appear better than they are.

**Blocked design:**
- Each treatment appears once on each shelf.
- All equally lucky.
- Comparisons made after taking out block effects.

<table>
<thead>
<tr>
<th>Experiment 1: completely randomised</th>
<th>Experiment 2: blocked</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Shelf 1</strong></td>
<td>Block 1 = Shelf 1</td>
</tr>
<tr>
<td>T1</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>T1</td>
</tr>
<tr>
<td><strong>Shelf 2</strong></td>
<td>Block 2 = Shelf 2</td>
</tr>
<tr>
<td>T2</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>T3</td>
</tr>
</tbody>
</table>
**BLOCKING**

**Selection of blocks:** often reflects the physical structure of the experimental units, or the physical process of the experimental procedure, e.g.

- morning/afternoon sessions.
- shelves within CE cabinet.
- whole CE cabinets.
- trays of plants.

- Treatments randomised to units **within each block** separately

**EXAMPLES**

a) **Field characteristics:** a slope, or fertility or pH trends across a field, or local pest problems.

Blocks are usually formed from sets of contiguous plots that are expected to be similar in as many respects as possible.
b) **Glasshouse characteristics:** differential shade or temperature due to positioning with respect to walls and doors are common in glasshouses. Blocks are usually formed from sets of trays or pots placed close together and hence in similar environmental conditions.

c) **Time of measurement:** some experiments may be processed over a lengthy period, and time of measurement may have a systematic effect on results. A set of units processed within the same time period can be considered as a block.
d) **Observer/scientist:** for subjective measures, such as visual scores, it is well-known that individuals will perceive scores differently.

If several different observers are scoring material or carrying out a laboratory process, then it makes sense to regard each person as a block.

e) **Batches of chemical/reagent, of plants, or of other organisms such as insects:** if there is any possibility of differences between batches, then they should be considered as a blocking factor.

f) **General structure:** natural structure in experimental material. All of these levels of structure should be considered as possible blocking factors.

For example, trays of plants may be held on shelves within a controlled environment cabinet.
• Variation is controlled and estimates of background noise reduced

• Block variation can be separated from background variation

  increased precision of treatment comparisons

Variability due to:
- magenta: treatment
- light blue: background
- dark blue: block

No blocking:

Blocking: