Culturing of *Daphnia magna* - Standard Operating Procedure

Introduction

The objective of this SOP is to culture *D. magna* under optimum conditions for maximum production of neonates. Methods of culture have been adapted from SOP No. 120/3 obtained from the Water Research Centre (WRC), Medmenham, UK.

Daphnia magna Straus (Cladocera) was obtained from WRC having originated from the National Institute for Applied Chemical Research (IRCHA), France. Our strain has been categorised as IRCHA Clone Type 5, by the *Daphnia* group at Sheffield University, and has been cultured since March 1999 at the University of Reading (Callaghan group).

Under ideal environmental (laboratory) physicochemical conditions *D. magna* reproduce parthenogenetically producing clonal offspring. However, a change in e.g. temperature or food levels may induce production of males with subsequent sexual reproduction and production of resting eggs (ephipia). Thus, production of males may be used as an indicator of changing conditions (e.g. stress); males should not be present in a laboratory culture reared under a regime of constant light, temperature and food.

At 20ºC *D. magna* reach sexual maturity in 6-8 days releasing their eggs into a brood chamber. The embryos complete their development inside the brood chamber and hatch as free-swimming neonates at day 8-10. In the following 2-4 days the mature females release a 2nd brood of neonates with reproduction peaking around the 3rd brood (day 12-14) or 4th brood (day 14-17). As the adult daphnids become older the time between broods will increase and the size of the brood will decrease. Even under constant culturing conditions, brood size may vary due to e.g. water quality and crowding (see below).

The parthenogenetic mode of reproduction (isolating genetic variability) and short life cycle (egg to adult in ~10 days) make *D. magna* an ideal organism for studying environmental stress responses from the molecular to population level.

Culture set-up and maintenance

When starting to culture *D. magna*, the very first cultures are initiated with 15 (<24 h old) neonates per vessel. These initial neonates must originate from a single female in order to minimise clonal variations such as age at maturity and brood size. Future cultures are initiated with 15 (<24 h old) neonates per vessel originating from a pool of offspring collectively produced by the parental cultures (NB! new cultures should only be started from neonates produced by age-synchronised adults). New cultures are set-up every 2-3 weeks and should ideally be started with 3rd to 5th brood neonates. Starting the cultures with ealier broods may reduce production, whereas using later broods may result in genetic drift.

The OECD recommends that cultures/experiments should not be initiated with 1st brood. (http://www.oecd.org/dataoecd/17/63/1948277.pdf).

D. magna (15 individuals) are cultured in 2 L plastic beakers containing 1.2 L ISO water (see appendix A) with, depending on the age of the daphnids (see example of culturing history in

Appendix C), 3 or 4 ml of organic additive 'Marinure' solution (Appendix B). NB! Add Marinure (mix well) and food (see below) before adding the daphnids.

Vessels are covered with cling-film or a Perspex disk to minimise evaporation and to reduce contamination.

All cultures are maintained under static conditions at 20±1ºC and a controlled photoperiod at 16 hrs light and 8 hrs darkness.

Cultures should be examined daily and diseased or dead individuals removed. Males and/or ephipia must also be removed if encountered. NB! A shift to sexual reproduction could be due to a change in temperature, photoperiod and/or food level/quality. Thus, as a means of quality control, it is recommended that minimum/maximum temperature be recorded daily (or at *least* weekly), and that photoperiod timers and algae cultures are checked regularly.

Daphnids are fed once daily (Monday-Friday) with a distilled suspension of *Chlorella vulgaris* 0.5-2.0 ml (see Appendix C and SOP on *C. vulgaris*). The algae ration is increased as the daphnids mature, and is kept constant once they reach adulthood (Appendix C). The algae food is supplemented with 0.5 ml per culture per day of a 100 mg/l stock suspension of dry baker's yeast (Appendix D).

Neonates must be removed daily before feeding (Monday-Friday) to avoid crowding and to ensure that the founding adults obtain a constant level of food. Neonates are removed either with a plastic pipette or when changing water in the cultures. A record of neonate production is recommended, in order to monitor the health of the founding adults and for comparison with historical records. All unused neonates are discarded down the sink or preferably used as fish food.

The ISO water should be changed on a weekly basis (minimum) to ensure optimal water chemistry. Adults are transferred with a plastic pipette (tip cut off to accommodate their body size) to vessels containing fresh ISO media, algae, yeast and Marinure. Used vessels must be cleaned in detergent and left to rinse overnight in a sink with a slow-running tap.

Materials and Methods

Labelling

- Organism name
- Date of initiating culture
- Operator's initials

Apparatus

- Dissolved Oxygen Meter
- Conductivity Meter

- pH Meter
- Digital thermometers
- Water hardness (Appendix A)

Record keeping, reporting, storage and retrieval

- Feeding, water change and general culture health parameters are recorded on data sheets and archived.
- Copies of Study Plans and SOPs should be circulated to all staff involved.
- Daphnids used for transcriptomic studies are stored in RNA*later*® at –80ºC, whereas unused individuals are discarded.

Appendix A: Media for culturing of *Daphnia magna* (modified after the ISO standard protocol)

- Weigh out the stated quantities of the following salts per 1 L of wanted media volume:

  195.87 mg $CaCl_2 \cdot 2H_2O$ (Calcium chloride)

  82.20 mg $MgSO_4 \cdot 7H_2O$ (Magnesium sulphate)

  64.80 mg $NaHCO_3$ (Sodium hydrogen carbonate)

  5.80 mg KCl (Potassium chloride)

  0.002 mg Na_2SeO_3 (Sodium selenite)*

  *Prepare a stock solution with a concentration of 40 µg/ml (1 year shelf life)

- Dissolve the calcium chloride separately in 300-500 ml reverse osmosis (RO) water using a magnetic stirrer.
- Add the calcium chloride solution to the required volume of RO water before continuing.
- Dissolve the remaining salts together in 300-500 ml RO water using a magnetic stirrer.
- Add the dissolved salts to the required volume of RO water and mix.
- Leave media a minimum 12 hrs to ensure proper mixing of the salt and keep it aerated.

- Measure pH, conductivity and water hardness before usage to ensure appropriate water chemistry:
    - pH should be between **pH 7.5 to pH 8.2**
      Conductivity should be between **360 to 480 µS*/cm** (*microsiemens)
- Water hardness should be between **130 to 160 mg/l** (measured as $CaCO_3$) and can be measured by titration (http://en.wikipedia.org/wiki/Titration):
    - Add 100 ml of prepared culturing media to a beaker/conical flask.
    - Add 5 ml Buffer Solution Ammonia-Ammonium Chloride (Fisher Scientific, catalogue# J/2500/17) to the beaker.
    - Add 10 droplets from a saturated Eriochrome Black T indicator solution (Fisher Scientific, catalogue# 22836-0250) to the beaker.
    - Add X ml of titrant, 0.01 M EDTA (Fisher Scientific, catalogue# J/3720/17), to the beaker using a 25 ml burette.

    - Note the exact volume of titrant needed for the colour of the analyte solution to change from purple to blue (1 ml of titration buffer equals 10 mg $CaCO_3$/l, e.g. 15.3 ml equals a water hardness of 153 mg/l).

- Record the batch number of media (label batches chronologically as they are prepared), water chemistry data, date of preparation and operator initials.

- Culturing media may be used for up to one month following preparation after which it should be discarded.

Appendix B: Preparation of nutritional supplement for *Daphnia magna* cultures

Marinure, a standard organic extract, can be purchased from Glenside Organics (http://www.glensideorganics.co.uk/).

- To obtain 1 L Marinure stock dissolve approx. 9-10 ml of the concentrated extract (remove using a Gilson5000 pipette) in 1 L of distilled water (shake the solution vigorously till the extract is fully dissolved).

- Make a 1:10 dilution of the Marinure stock (e.g. 1.8 ml distilled water to 0.2 ml dissolved marinure) in a disposable plastic cuvette.

- Measure optical density of the 1:10 diluted sample (OD1:10) using a spectrophotometer (remember to use a blank control cuvette containing 2 ml distilled water).

- At a wavelength of 400 nm the OD of the 1:10 diluted sample should read 0.800 (±5%, i.e. OD1:10 should be within 0.760 and 0.840).

- If the reading is above or below OD1:10 = 0.800 (>5%) then add a bit more extract or add a bit more distilled water to the stock, respectively.

- Make a new 1:10 dilution of the modified Marinure stock (after adding more extract or water) in a new plastic cuvette (use same blank control).

- Measure OD1:10 of the modified stock. Continue as above till spectrophotometric measurement reads 0.800 (±5%).

- Label the stock Marinure, e.g.
  Marinure OD1:10(400nm) = 0.800
  Preparation date
  Expiry date (6 months shelf life)
  Initials

- Add 3 ml Marinure to 1.2 L culture media when starting/renewing daphnia culture media containing daphnids <7 days old.

- Add 4 ml Marinure to 1.2 L culture media when starting/renewing daphnia culture media containing daphnids >7 days old.

Appendix C: An example of culturing history

Daphnia magna - Culture Data Sheet

Initiation Date 01/03/2006 **Operators initials** LHH **Culture reference#** 21A-C

Date	Day	Culture (# founding ind.)			#Offspring			Food*		Water renewal		Remarks
		A	B	C	A	B	C	Algae (ml)	Yeast (ml)	Batch#	Marinure (ml)	
01/03	1	15	15	15				1.0	0.5	32	3.0	
02/03	2							1.0	0.5			
03/03	3							1.5	0.5			50% more food on Fridays
04/03	4											Weekend no feeding
05/03	5											Weekend no feeding
06/03	6							1.5	0.5			
07/03	7							1.5	0.5			
08/03	8	15	15	15				2.0	0.5	32	4.0	Water changed
09/03	9				<100	<100	<100	2.0	0.5			1st brood
10/03	10				<100	<100	<100	3.0	0.5			50% more food on Fridays
11/03	11											Weekend no feeding
12/03	12											Weekend no feeding
13/03	13				~200	~250	~200	2.0	0.5			Second brood removed
14/03	14	15	15	14	<100	<100	<100	2.0	0.5	32	4.0	Water changed, neonates removed
15/03	15							2.0	0.5			No juveniles at 14:30
16/03	16				~250	~300	~200	2.0	0.5			New cultures started (3rd brood)
17/03	17							3.0	0.5			50% more food on Fridays
18/03	18											Weekend no feeding
19/03	19											Weekend no feeding
20/03	20				>300	>300	>300	2.0	0.5			3rd/4th brood removed
21/03	21	15	15	14	<100	<100	<100	2.0	0.5	33	4.0	Water changed
22/03	22							2.0	0.5			
23/03	23							2.0	0.5			
24/03	24											Discarded (new cultures reproducing)

Yeast is added at a constant rate of 0.5 ml (100 mg/l) throughout, whereas the ration of algae is increasing until the daphnids reach adulthood: 1.0 ml algae on day 1-2; 1.5 ml algae on day 3-7; 2.0 ml algae on day 8+ (NB! 1 ml algae is equivalent to 0.50 mg carbon at OD1:10(440nm) = 0.800 - for further information see SOP on *Chlorella vulgaris*). Note that algae rations are increased by 50% on Fridays to compensate for no feeding during the weekend.

Appendix D: Preparation of baker's yeast stock solution for *Daphnia magna* cultures

100 mg/l baker's yeast stock:

- Weigh out ~10 mg of dry baker's yeast and transfer it to a blue-cap bottle.
- Add 100 ml of distilled water.
- Use magnetic stirrer to "speed up" the dissolution of the yeast.
- 0.5 ml of yeast stock solution (100 mg/l) is added daily (Monday-Friday) to cultures.
- Renew stock every 1-2 weeks.