**Negative relationships between population density and metabolic rates are not general**

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Running headline: Density-dependent metabolic rate

**Summary**

1. Population density has recently been suggested to be an important factor influencing metabolic rates, and to represent an important ‘third axis’ explaining variation beyond that explained by body mass and temperature. In situations where population density influences food consumption, the immediate effect on metabolism acting through specific dynamic action (SDA), and downregulation due to fasting over longer periods, is well understood. However, according to a recent review, previous studies suggest a more general effect of population density *per se*, even in the absence of such effects. It has been hypothesised that this results from animals performing anticipatory responses (i.e. reduced activity) to expected declines in food availability.
2. Here we test the generality of this finding by measuring density effects on metabolic rates in 10 clones from two different species of the zooplankton *Daphnia* (*Daphnia pulex* Leydig and *D. magna* Straus). Using fluorescence-based respirometry we obtain high-precision measures of metabolism.
3. We also identify additional studies on this topic that were not included in the previous review, compare the results, and evaluate the potential for measurement bias in all previous studies.
4. We demonstrate significant variation in mass-specific metabolism among clones within both species. However, we find no evidence for a negative relationship between population density and mass-specific metabolism. The previously reported pattern also disappeared when we extended the set of studies analysed.
5. We discuss potential reasons for the discrepancy among studies, including two main sources of potential bias (microbial respiration and declining oxygen consumption due to reduced oxygen availability). Only one of the previous studies gives sufficient information to conclude absence of such biases, and consistent with our results no effect of density on metabolism was found. We conclude that population density *per se* does not have a general effect on mass-specific metabolic rate.

**Key-words:** Abundance, competition, digestion, genetic variation, metabolic theory of ecology, oxygen consumption, respiration

**Introduction**

Metabolic rate is a highly plastic trait that responds strongly to a range of individual characteristics (Marhold & Nagel 1995; Gillooly *et al.* 2001; Vezina, Speakman & Williams 2006; Killen, Atkinson & Glazier 2010) as well as environmental factors (Gillooly *et al.* 2001; Millidine, Armstrong & Metcalfe 2006; Scantlebury *et al.* 2007). Recently, DeLong, Hanley & Vasseur (2014) highlighted population density as an important contributor to determining metabolic rates, with an emphasis on the negative influence of population density on *per capita* resource availability, and a corresponding decrease in metabolism. The physiology behind this is well understood; an immediate effect of lower food consumption at higher density is to reduce metabolism via effects on specific dynamic action (SDA, i.e. additional energy expenditure due to the costs of processing food, including digestion, absorption, nutrient distribution and synthesis of new tissue, Ikeda 1977; Kiørboe, Møhlenberg & Hamburger 1985; Lampert 1986; Urabe & Watanabe 1990), and over longer time periods of fasting additional downregulation is expected (e.g. Hervant, Mathieu & Durand 2001).

DeLong *et al.* (2014) presented a range of studies (their Table 1) demonstrating negative relationships between density and mass-specific metabolism. The majority of these relationships were estimated in the presence of food, and hence when SDA can be expected to respond to population density. However, for some of the studies, including one on the cladoceran *Daphnia ambigua*, metabolic rate decreased with increasing density when food resources were not available, i.e. when reduced SDA should not be expected. It is not obvious how density should influence metabolism if *per capita* resource availability is kept constant. DeLong *et al.* (2014) suggested that a possible explanation for the negative relationship between density and metabolic rate in the absence of food is that organisms respond to increased density by reducing their activity. Thus, in the absence of direct effects of density on food resources this would entail an anticipatory response to offset expected reductions in food abundance. This would explain their results for *Daphnia* as well as other studies performed in the absence of resources. Here we test the generality of this finding by conducting a similar experiment using *D. pulex* and *D. magna*, two species that are ecologically similar to *D. ambigua*. DeLong *et al.* (2014) found a tendency for differences in density response among clones that they tested, and we therefore included five different clones from each species. Our results differ markedly from those of DeLong *et al.*, and we discuss potential reasons for this discrepancy. Furthermore, we identify additional data sets quantifying responses of metabolism to density in the absence of resources, compare the results from these with those presented by DeLong *et al.*, and evaluate to what extent previous studies give sufficient information to conclude the absence of measurement biases.

**Materials and Methods**

*Study animals and husbandry*

Five clones of *Daphnia pulex* were hatched in 2013 from ephippia collected in Lake Asklundvatnet (4.0 ha, 63.588°N, 10.729°E), central Norway, and five clones of *Daphnia magna* were hatched in December 2014 from ephippia collected in a pond at Værøy Island (1.0 ha, 67.687°N 12.672°E), northern Norway. *Daphnia* were cultured at 17°C with a 16L:8D photoperiod in ADaM medium (Klüttgen *et al.* 1994, SeO2 concentration reduced by 50%). These were fed three times a week with Shellfish Diet 1800 (Reed Mariculture Inc, USA) at a final concentration of alga 2x105 cells ml-1 for *D. pulex*, and 4x105 cells ml-1 for *D. magna*. Medium was exchanged weekly.

*Respirometry method*

Respiration was measured in a sealed glass micro plate equipped with planar oxygen sensor spots with optical isolation glued onto the bottom of 200 μL wells (Loligo Systems, Denmark) integrated with a 24-channel fluorescence-based respirometry system (SDR SensorDish® Reader, PreSens, Germany). Such optode respirometry is known for its simplicity, high throughput, and high temporal resolution and sensitivity (Szela & Marsh 2005). The reader was placed inside a Memmert Peltier-cooled incubator IPP (Memmert, Germany) at 17°C, which was also used for bringing all equipment and ADaM to the same temperature prior to measurements. *Daphnia* were rinsed in ADaM and transferred into wells containing air-saturated ADaM. Wells were inspected for air-bubbles, overfilled with ADaM and sealed before the plate was placed on the SDR Reader. Preparation of the plate was conducted in a steel container placed in a 17°C water bath to minimize changes in temperature.

The wells in this system are delivered with screw caps for sealing. However, we did not use these for two reasons. First, they prevent inspection of wells after sealing, making it impossible to inspect wells for air bubbles or observing the animals. The manufacturer’s recommendation to seal the wells under water to prevent air bubbles is not feasible for small organisms. Second, because the sensors are highly sensitive to pressure, sealing with these caps introduces considerable noise into measurements due to variation in the resulting pressure once sealed. This problem becomes even bigger if subsequent changes in temperature (and hence pressure) occurs within wells after sealing. By performing a series of pilot experiments an alternative method for sealing was developed, using transparent adhesive PCR film (Thermo Scientific, USA, Appendix S1).

Following sealing of wells, measurements of oxygen concentrations were taken every 3 min. during 1.5 hour periods in darkness using the SDR v38 Software (PreSens, Germany). The optimal measurement interval based on evaluation of pilot experiments results was found to be 20-80 minutes after the sealing of wells. Using this interval produced linear declines in oxygen concentration (i.e. no temporal change in consumption during this period) and low uncertainty in consumption estimates (Appendix 2). Thus, data from this period were used for all runs. Total oxygen consumption by *Daphnia* was calculated while correcting for observed changes in oxygen readings in control wells (i.e. wells without animals). Finally, although our method for sealing the wells avoids effects of pressure changes, it allows for some diffusion of oxygen into the wells. However, this was also accounted for when calculating oxygen consumption (Appendix S3).

*Experimental designs*

Tests of density-dependent respiration generally falls into two design categories (Table 1), where the first one exposes organisms to different densities during respirometry, and the second one keeps organisms at different densities for an extended period prior to respirometry performed at a common density. The former one is the one most commonly applied (Table 1), and is therefore the one we apply most extensively in the current study, where we test for both variation in responses among species and among clones within species. However, we also provide an additional smaller data set where we test for effects of density treatments prior to respirometry in one of the species (*D. magna*).

*Effects of density during respirometry*

To test for effects of density during respirometry, juvenile individuals from second or older clutches were transferred individually into spot-plates (3.5 ml wells) containing ADaM and starved for 18 hours prior to the experiment to minimize oxygen consumption due to specific dynamic action. To estimate density effects one to four individuals from a single clone were randomly allocated to 20 of the 24 wells. For each species we conducted one run per clone for a total of 10 runs (2 species × 5 clones). In each run, the remaining four wells were used as controls (i.e. only containing ADaM medium).

*Effects of density prior to respirometry*

To test for effects of having been kept at different densities over a longer period, juveniles from second or older clutches were kept at two different densities for 66 hours prior to measurements. For this experiment we only used *D. magna*. Individuals of all five clones of *D. magna* were mixed before being allocated haphazardly to twenty-seven 70 ml culture bottles. Five bottles contained 28 individuals, whereas the remaining 22 bottles contained single individuals. Using a 16-channel peristaltic pump, each bottle received a constant flow of 120 ml h-1 from a common reservoir containing 25 l aerated ADaM and Shellfish diet at a concentration of 2 x105 alga cells ml-1. The outflow of each bottle was circulated back into the reservoir. Thus, using a high flow rate relative to the filtration rate of daphnia (Burns 1969) we prevented effects of crowding on food availability, and the circulating system allowed all bottles to receive medium containing metabolites. To fit all bottles to the same peristaltic pump, the 22 single individual bottles were attached successively in pairs to eleven of the channels. The five crowded bottles were attached to the remaining five channels. The experiment was run at 17 ˚C. During the final 18 hours of the experiment the medium in the reservoir was replaced with pure ADaM to minimize oxygen consumption due to specific dynamic action during respirometry. Respirometry was performed as described above, except that each well only contained single individuals. Individuals from the two treatments were randomly allocated to 20 of the 24 wells in each of two replicate respirometry runs, with the remaining four wells being controls. Due to some mortality and accidental losses the final sample sizes were N = 16 and 19 for the individual and crowded treatments, respectively.

*Clone effect*

A third experiment was performed to test for variation in respiration among clones (within species) at a given constant density. For this, respiration of single individuals from all five clones of one species was measured in the same run. Four replicate runs were conducted per species. Thus, this design allows for estimates of clone effects while accounting for run effects. As for the density effect experiment, wells with ADaM were used as controls. In each run, four wells were used as controls.

*Length and Mass Determination*

Following respirometry, individuals were photographed under a stereomicroscope and their carapace length (BL, mm) was measured using ImageJ 1.44p software (National Institutes of Health, Bethesda, MD). To allow dry weight estimations from these lengths, a sample of each species were starved for 24h (to allow stomach evacuation), photographed, fixed using 70% ethanol, and had their eggs (if any) removed. They were then transferred individually into weighing foil trays, dried overnight at 60°C and weighed on a Mettler Toledo UMX 2 microbalance. Body length ranges for these samples were 0.70 – 2.86mm and 0.94 – 3.92mm for *D. pulex* and *D. magna*, respectively. The relationship between BL and dry mass (DM, mg) was estimated for *D. pulex as DM* = 0.00402*BL*2.66 (df = 36, r2 = 0.92, P < 0.001) and for *D. magna* as DM= 0.00535*BL*2.72 (df = 30, r2 = 0.99, P < 0.001).

*Statistical analyses*

All statistical analyses were conducted using R v. 2.9.2. (R Development Core Team). For the experiment testing for effects of density during respirometry the mass specific oxygen consumption rate in each well (mg O2 h-1 mg DM-1) was first calculated and log-transformed to homogenize residual variances. The two species were treated separately in the subsequent analyses. The model included log mass specific oxygen consumption rate as response variable, and density (number of individuals, covariate) and run (factor) as well as their interaction as predictor variables. In this experiment run is confounded with clone, preventing us from estimating the specific clone effects. We note, however, that the interaction between run and density will reflect clone differences in their response to density.

For the experiment testing for effects of density prior to respirometry, we first tested for effects of position of the bottle in the single individual treatment (i.e. being first or second within a pair). The model included log oxygen consumption rate (mg h-1) as response variable, and individual dry mass (mg, covariate), position (factor) and respirometry run (factor) as fixed predictor variables, and peristaltic pump channel as a random factor. Model selection (for procedure see below) based on this reduced data set showed that bottle position within channel had no effect (*P* = 0.671). Thus, when testing for effects of crowding treatment on log oxygen consumption rate, the full model contained individual dry mass (covariate), treatment, run (fixed factor) and channel (random factor), whereas position of bottles within pairs of single individual treatments was not included.

For the clone effect, we fitted models where the log oxygen consumption rate (mg h-1) was the response variable, and individual dry mass (mg, covariate), and clone and run (fixed factors) were independent variables. The full model also included an interaction between dry mass and clone. For this data set all clones were represented in each run, thus enabling separate estimates of these two effects.

The models were implemented using the *lme* and *gls* functionsin the package *nlme* (Pinheiro *et al.* 2009). Model selection was conducted using a backwards selection procedure where variables were removed sequentially until no further model simplification could be made without causing a significant decrease in log-likelihood (calculated based on maximum likelihood, Zuur *et al.* 2009).

**Results**

*Effects of density during respirometry, D. pulex*

The interaction between run and number of individuals could be removed without causing a significant reduction in log-likelihood (*P* = 0.777). Neither run nor density could be removed as main effects without causing a significant reduction in log-likelihood (*P* < 0.001 and *P* = 0.010, respectively). However, the estimated slope for the density effect was weak and positive rather than negative (0.06 ± 0.02SE, corresponding to a ca. 6% increase when increasing number of individuals by one, Fig. 1a).

To estimate the amount of explained variation in respiration using our method we also fitted a linear model with log absolute oxygen consumption rate (mg h-1) as response variable, and log total dry biomass (mg) and run as independent variables. This model explained 87% of the variation in respiration (Fig. 2a). The estimated slope (± SE) of the biomass effect was 1.09 ± 0.05.

*Effects of density during respirometry, D. magna*

The interaction between run and number of individuals could not be removed without causing a significant reduction in log-likelihood (*P* = 0.048). When fitting separate models for each run, two estimated effects of number of individuals were negative and three were positive, but none of these were significant when adjusting p-values using a sequential Bonferroni approach (mean ± SE, -0.05 ± 0.04, -0.02 ± 0.04, 0.05 ± 0.07, 0.08 ± 0.04, 0.11 ± 0.04, *P* > 0.05 for all). Thus, no clear overall pattern could be observed between oxygen consumption and number of individuals (Fig. 1b).

A linear model with log absolute oxygen consumption rate (mg h-1) as response variable and log total dry biomass (mg) and run as independent variables explained 89% of the variation in respiration (Fig. 2b). The estimated slope (± SE) of the biomass effect was 0.99 ± 0.05.

*Effects of density prior to respirometry*

Both channel (*P* = 0.225), run (*P* = 0.998) and crowding treatment (*P* = 0.150) could be removed from the model of oxygen consumption of individuals having been kept at different densities prior to respirometry. Thus, only dry body mass remained as having a significant effect in the final model (*P* < 0.001). When adjusting to the common mean body mass of 0.021 mg, the mean (SD) oxygen consumption was 0.19 (0.05) and 0.16 (0.03) µg h-1 for the single individual and crowded treatments, respectively.

*Clone effect, D. pulex*

Both the run effect (*P* = 0.760) as well as the interaction between clone and body mass (*P* = 0.703) could be removed without causing a significant reduction in log-likelihood. Neither clone nor body mass could be removed as main effects without causing a significant reduction in log-likelihood (*P* < 0.001 for both). The clone with the highest size adjusted oxygen consumption had a mean value that was 83% larger than the clone with the lowest consumption (Fig. 3a).

*Clone effect, D. magna*

Both the run effect (*P* = 0.445) as well as the interaction between clone and body mass (*P* = 0.094) could be removed without causing a significant reduction in log-likelihood. Neither clone nor body mass could be removed as main effects without causing a significant reduction in log-likelihood (*P* = 0.002 and *P* < 0.001, respectively). The clone with the highest size adjusted oxygen consumption had a mean value that was 26% larger than the clone with the lowest consumption (Fig. 3b).

**Discussion**

In the present study we tested for a relationship between population density and metabolic rates in *D. pulex* and *D. magna*. Our experimental method, using an optical fluorescence-based respirometry system in combination with a novel method for sealing, yielded high quality data that allowed close to 90% of variation in oxygen consumption to be explained by variation in total dry body mass across wells containing one to four juveniles. Furthermore, significant variation in individual metabolism could be detected among clones within species with relatively modest sample sizes (6-12 replicates per clone). Thus, our method for measuring metabolism was able to accurately describe sources of variation. Yet, in contrast to previous reports (DeLong *et al.* 2014) there was no overall negative relation between population density and mass-specific oxygen consumption in either of the two species. This was also true for the experiment using *D. magna* where we manipulated density for an extended period prior to respirometry. Furthermore, evidence for variation among clones in the density response, as reported in an experiment on *D. ambigua* by DeLong et al., was weak. No such variation was observed in *D. pulex*, whereas an interaction between density and run (and hence clone) was only marginally significant for *D. magna* (P = 0.048), and none of those clones had a significant negative relationship.

The current study demonstrates that neither *D. pulex* nor *D. magna* have a lower mass-specific metabolic rate at higher population density in the absence of effects on food abundance. The discrepancy between our conclusion and the results obtained by DeLong *et al.* on *D. ambigua* may be caused by species differences. Such species-specific patterns may be expected when comparing species with different social structure. For example, in organisms with high intensity of interference competition, higher density may lead to higher levels of activity metabolism, and even higher total metabolism (Li & Brockesen 1977). Conversely, higher density may lead to decreased total metabolism in organisms that naturally live in groups due to reduced activity (Waters *et al.* 2010) and/or a physiological ‘calming effect’ (i.e. due to reduced perceived predation risk, Schleuter *et al.* 2007). However, it is not clear why the *D. ambigua* used by DeLong *et al.* would respond differently to population density than the two species we used in our study. All these species are ecologically similar, being zooplankton that feed by filtering out algae and bacteria from the water of lakes and ponds, and hence show little potential for direct social interactions. An exception to this is during the formation of swarms, which may be triggered both by predation risk (Pijanowska & Kowalczewski 1997) and food abundance (Kleiven, Larsson & Hobæk 1996), but it is not known to what extent such behaviour differs among the species discussed here, and if they differ how this would influence the optimal regulation of metabolism in response to density.

An alternative explanation for the different results is an influence of some methodological aspect that introduces a bias into metabolism measurements with respect to density. At least two potential sources of such a bias may exist. First, it is vital that all oxygen consumption that is used to calculate *per capita* consumption is due to metabolism of the focal organism. If microbial consumption is erroneously assigned to the focal organism’s consumption, the latter will be overestimated, and more so for the lower density treatments. Thus, failing to account for such consumption will lead to a bias, causing a spurious negative relation between density and metabolism. Second, many organisms including *Daphnia* reduce their oxygen consumption when concentrations become sufficiently low (Heisey & Porter 1977; Weider & Lampert 1985). If this is the case in a given experiment, this will also lead to a bias towards reduced consumption for higher densities. We therefore evaluated the possibility for such a bias in existing studies testing for density-dependent metabolism in the absence of resources. In addition to the four studies given in DeLong et al. (2014) we identified another three studies of relevance. Whereas all studies reported by DeLong et al. reported negative relations between density and metabolism, none of the studies identified here do (Table 1). Furthermore, one of the studies cited by DeLong et al. that reported a negative relationship between density and metabolism was explained by the authors to be caused by reduced oxygen availability at high densities (Nässberger & Monti 1984). Based on the information given in the two other studies reporting negative relationships it was not possible to determine the potential for the two sources of bias identified above. Only one of the additional studies documented how such biases were avoided, with results suggesting no effect of density (Roff 1973, Table 1). In our experiments we accounted for both sources of bias by the use of blanks (wells without *Daphnia*) to control for microbial consumption, and by ensuring that oxygen consumption was constant during our measurement interval (i.e. linear decline in oxygen concentrations, Appendix S2). Due to the common lack of information about how possible biases were accounted for it is often challenging to compare results of different studies. We therefore urge future studies on respiration to be careful to avoid such biases, and to present sufficient experimental detail to enable readers to evaluate this.

In conclusion, our results demonstrate that, barring the more obvious effect of density on metabolism when density influences food consumption, density *per se* does not have a general effect on metabolism. It is possible that there is variation in such responses even among ecologically similar species, for example, due to differences in how they react to density behaviourally, but this remains to be demonstrated in controlled experiments where a common approach is used across species and where experimental bias in metabolic rate measurements is avoided. Such knowledge would be valuable for our understanding of how population dynamics is linked to physiological and behavioural traits.

**Data Accessibility**

Data available from the Dryad Digital Repository: doi:10.5061/dryad.42tr5

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Table 1. Studies testing for density-dependent metabolism in the absence of food resources. NA indicates that publications do not contain the required information. The first four entries are those given by DeLong et al. (2014). Studies are divided into two categories; organisms were either exposed to different densities during respirometry (category I), or to different densities for a period prior to respirometry performed at a common density (category II). Duration of density treatment (i.e. duration of measurement for category I or duration of pre-measurement exposure for category II) are given in brackets. N - range in number of individuals, V- volume (ml) of container used during measurements.

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| --- | --- | --- | --- | --- | --- | --- | --- |
| Organism | Density-dependent metabolism | Category | Prevent microbial respiration bias | Prevent oxygen availability bias | N | V | References |
| *Daphnia ambigua* | Negative | I (NA) | NA | NA | 1-4 | 0.6 | DeLong *et al.* 2014 |
| *Amoeba proteus* | Negative | II (3-5 d) | No | No\* | 1500-10000 | NA | Nässberger & Monti 1984 |
| *Scenedesmus obliquus* | Negative | II (NA) | NA | NA | NA | 1 | Walsh *et al.* 2012 |
| *Simocephalus vetulus* | Negative | I (1-12 h) | NA | NA | 250 - 2000 | 250 | Hoshi 1957 |
| *Daphnia pulex* | 0 | I (10 h) | Yes | NA | 2-8 | 4 | Goss & Bunting 1980 |
| *Daphnia pulex* | Positive | I (1 h) | Yes | Yes | 1-4 | 0.2 | Present study |
| *Daphnia magna* | 0 | I (10 h) | Yes | NA | 1-6 | 4 | Goss & Bunting 1980 |
| *Daphnia magna* | 0 | I (1 h) | Yes | Yes | 1-4 | 0.2 | Present study |
| *Daphnia magna* | 0 | II (66 h) | Yes | Yes | 1-28 | 70 | Present study |
| *Limnocalanus macrurus* | 0 | I (2-60 h) | Yes | Yes\*\* | 3-54 | 30 | Roff 1973 |
| Zooplankton\*\*\* | Positive | I (1 h) | No | NA | NA | 470 | Satomi & Pomeroy 1965 |

\*Metabolism was measured by calorimetric methods, and negative relationship suggested by authors to result from reduced oxygen availability

\*\*Final oxygen concentrations never less than 80% of initial values

\*\*\*Marine zooplankton samples of mixed taxonomic composition collected from the wild



Figure 1: Dry mass specific oxygen consumption rate (mean ± SE) of (a) *D. pulex* and (b) *D. magna* measured at different densities. To visualize the effects of density, residuals were obtained from a model containing only run effects, and these were added to the overall mean consumption rate to obtain appropriate scale. N = 11 - 26 (total 84) for the different treatments for *D. pulex*, and 21 - 25 (total 91) for *D. magna*.



Figure 2: Log oxygen consumption rate of (a) *D. pulex* and (b) *D. magna* as a function of log total body dry mass. To visualize the effects of body mass, residuals were obtained from a model containing only run effects, and these were added to the overall mean consumption rate to obtain appropriate scale.



Figure 3: Oxygen consumption rate (mean ± SE) of different clones of (a) *D. pulex* and (b) *D. magna* when measured individually. To visualize the effects of clone, residuals were obtained from a log-linear model containing only dry body mass, and these were added to the overall mean consumption rate to obtain appropriate scale (i.e. data are size adjusted to the mean size within species, mean (SD), *D. pulex* 0.010 (0.002) mg, *D. magna* 0.034 (0.008) mg. N = 10 - 12 (total 57) for each clone for *D. pulex*, and 6 - 8 (total 38) for *D. magna*.