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The effect of *Pseudo-nitzschia seriata* on grazing and fecundity of *Calanus finmarchicus* and *Calanus glacialis*

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This study investigates whether feeding on the domoic acid (DA)-producing diatom *Pseudo-nitzschia seriata* affects the faecal pellet (FP) production (proxy for grazing) and fecundity of *Calanus finmarchicus* and *Calanus glacialis*. Female copepods were fed a saturating concentration of food (400 μg C L^{-1}) in two combinations (i) natural phytoplankton spiked with 50% *P. seriata* and (ii) only the non-toxic phytoplankton community. The copepods ingested DA, as illustrated by DA accumulation in their FPs, and transferred a share of DA to their eggs and body tissue. DA was mainly excreted through FPs in both species. Compared with *C. finmarchicus*, *C. glacialis* accumulated substantially more DA in its body tissue. For both species, egg production and hatching success were unaffected by grazing on the toxic diatom. This suggests that feeding on DA-containing *P. seriata* does not affect copepod fecundity, despite increasing DA concentrations of *P. seriata* during the experiment.

KEYWORDS: *Calanus glacialis*; *Calanus finmarchicus*; fecundity; *Pseudo-nitzschia seriata*; domoic acid; toxin

INTRODUCTION

The pelagic ecosystem of the Arctic is characterized by a short period of high primary production (Levinsen *et al.*, 2000). When the ice cover opens in spring, light triggers the formation of a diatom-dominated phytoplankton bloom (Behrenfeld and Boss, 2014). Copepods of the genus *Calanus* are the primary herbivores during the spring bloom in western Greenland (Nielsen and Hansen, 1995) and represent a key group in the Arctic marine environment, since many species of fish, seabirds

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and marine mammals depend either directly or indirectly on these copepods (Falk-Petersen *et al.*, 1986, 2004; Dahl *et al.*, 2003). The dominant zooplankton species in Greenland waters are *Calanus finmarchicus, Calanus glacialis* and *Calanus hyperboreus* (Nielsen and Hansen, 1995). *Calanus glacialis* has a life cycle of 1–3 years and is a true Arctic species, with a distribution associated with Arctic water masses (reviewed in Falk-Petersen *et al.*, 2009). Its smaller and less-lipid-rich relative *C. finmarchicus* has a life cycle of 1 year and is a temperate species with a distribution corresponding to Atlantic water masses (reviewed in Falk-Petersen *et al.*, 2009). These copepods undergo diapause, i.e. they spend winter in hibernation in deep waters and ascend during spring in time to match the vernal phytoplankton bloom (Madsen *et al.*, 2001). *Calanus finmarchicus* and *C. glacialis* reproduction is linked to the spring bloom, since these copepods spawn near the surface during this period of high production (Hirche and Bohrer, 1987; Madsen *et al.*, 2001). They descend again in midsummer toward the end of the productive season (Madsen *et al.*, 2001).

The frequency and intensity of harmful algal blooms have increased on a global scale and there seems to be a shift toward toxic phytoplankton species (reviewed in Van Dolah, 2000). One group of toxic phytoplankton is the pennate diatom genus *Pseudo-nitzschia*. Around 30% (12 of 37) of all *Pseudo-nitzschia* species are able to produce domoic acid (DA), which is a neurotoxin that can be lethal to organisms and cause Amnesic Shellfish Poisoning (Tammilehto *et al.*, 2012; Trainer *et al.*, 2012). The first record of a DA-producing *Pseudo-nitzschia* species in the Arctic was *P. seriata* in western Greenland off the coast of Nuuk (Hansen *et al.*, 2011). Calanoid copepods grazing on toxic *Pseudo-nitzschia* species have been reported to accumulate DA, for example by concentrating DA within their digestive system as well as in their tissue (Lincoln *et al.*, 2001; Leandro *et al.*, 2010; Tammilehto *et al.*, 2012; Harðardóttir *et al.*, 2015). Moreover, it has been shown that some individuals of the endangered western North Atlantic Right Whale (*Eubalaena glacialis*) contained high concentrations of DA due to the ingestion of *C. finmarchicus* (Leandro *et al.*, 2010; Doucette *et al.*, 2012). Accordingly, *C. glacialis* and *C. finmarchicus* transfer DA to higher trophic levels (Tammilehto *et al.*, 2012). However, the effect of DA on the copepod itself is not clear yet (Tammilehto *et al.*, 2012). Nevertheless, if DA had a negative effect on the fecundity (i.e. egg production and egg hatching success) of these key Arctic copepod species, DA could cause zooplankton biomass to decline and thereby pose severe cascading effects to higher trophic levels with implications for the marine ecosystem and fisheries.

Here, we investigate the effect of grazing on a natural phytoplankton community spiked with the DA-producing diatom *Pseudo-nitzschia seriata* on the fecundity and FP production of the two calanoid copepod species *C. finmarchicus* and *C. glacialis* and aim to establish a better understanding of the fate of the ingested DA.

METHOD

Study site

Sampling was conducted 2 days prior to initiation of the experiments, on 28 April 2014 in Disko Bay near Qeqertarsuaq, approximately one nautical mile off the coast by RV "Porsild" (Arctic Station, Copenhagen University) at a 320-m deep station (69°13′N, 53°23′W; Fig. 1).

The experiment was conducted in a temperature-controlled container at the Arctic Station (University of Copenhagen) in Qeqertarsuaq (temperature 4 ± 1.5°C).

Sampling of copepods and phytoplankton

The copepods were collected by vertical hauls from the bottom to the surface using a WP-3 net (200 μm mesh size) with a large nonfiltering cod-end. Subsequently, the copepods were stored in a thermo box filled with sea water at *in situ* temperatures in the dark. Within 6 h of collection, adult *C. glacialis* and *C. finmarchicus* females were sorted with a wide-mouth pipette in ice-chilled Petri dishes filled with seawater under a stereo microscope (Leica Wild M3C) at the laboratory in accordance with size and pigmentation criteria defined by Nielsen *et al.* (Nielsen *et al.*, 2014). Then the females were transferred into 10 L buckets containing a cylinder (28.3 cm high and 18 cm in diameter) with an egg separator (400 μm mesh) containing 0.45 μm filtered seawater at 4 ± 1.5°C and starved for 1 day.

The natural phytoplankton community was collected at the same location from the depth of chlorophyll maximum at around 20 m using a 30 L Niskin water sampler.

Phytoplankton

The phytoplankton community was screened through a 100 μm filter to remove larger grazers without eliminating diatoms or larger phytoplankton species. Subsequently, it was diluted with 0.45 μm filtered sea water and transferred to 10 L polycarbonate containers and kept under the experimental conditions (12 h light:12 h dark, 9 ± 3 μmol photons m^{-2} s^{-1}, 4 ± 1.5°C).

Pseudo-nitzschia seriata strains had previously been isolated from the same location and were kept in culture at the Natural History Museum of Denmark, where they were grown in culture bags at irradiances of around

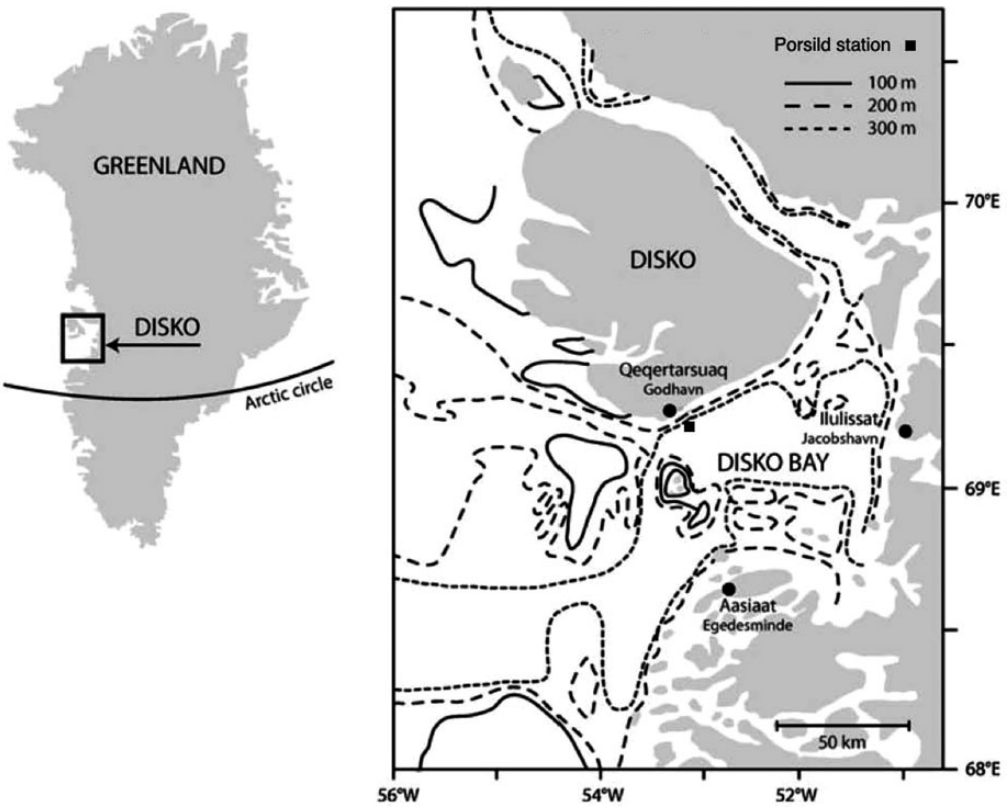

Fig. 1. Disko Bay on the west coast of Greenland where the sampling took place.

100 μmol photons m^{-2} s^{-1} at a temperature of 4 ± 1.5°C. The length (*L*) and width (*W*) of 20 *P. seriata* cells were measured using a light microscope (Olympus BX53, 20 × magnification) and a digital camera (Olympus DP72) using cellSens Entry software and then converted into the cellular volume (V, Table I) by following the formula based on Lundholm *et al.* (Lundholm *et al.*, 2004): $V = (0.6 \times L \times W^2) + (0.4 \times 0.5 \times L \times W^2)$. The carbon content of *P. seriata* was calculated according to Tammilehto *et al.* (Tammilehto *et al.*, 2012) based on the size and carbon content of *P. multiseries* of 0.12 pg C μm^{-3} which was then related to the measured volume of *P. seriata*, yielding an average cellular carbon content of 242 pg C $cell^{-1}$.

The carbon biomass of the natural phytoplankton community was estimated from cell counts dividing the sample into large cells (>6 μm width or diameter) and small cells (≤6 μm) based on visual observation and related to a rough estimate of carbon content of 231 pg C large $cell^{-1}$ and 18 pg C (small $cell^{-1}$) (Tammilehto *et al.*, 2012).

The size structure of the fixed (1% Lugol) natural phytoplankton community was analyzed as well. A sample of 15 cells of both size fractions was measured (Olympus DP72, 10× magnification, Table I). Three times during the experiment, a triplicate subsample (100 mL each) of the phytoplankton suspension from each treatment was removed and filled into 300 mL snow-chilled polycarbonate (Niskin) bottles for subsequent DA analysis.

Table I: Average size of phytoplankton fed to copepods [standard deviation (±) indicated]

	Cell length/ diameter (μm)	Cell width (μm)[a]	Volume (μm^3)
Small phytoplankton (≤6 μm)	4.8 ± 0.8	3.3 ± 0.3	64.6 ± 33.2[b]
Large phytoplankton (>6 μm)	23.1 ± 15.1	7.8 ± 3	3240.2 ± 544.4[c]
Pseudo-nitzschia seriata	80.3 ± 7	6.4 ± 1	2018.1 ± 577.4

[a]Shortest side of cell.
[b]Only spherical cells considered.
[c]Calculated assuming cylindrical shape.

Fecundity experiment

In total, 20 actively swimming *C. finmarchicus* females and 20 *C. glacialis* females were transferred individually with a wide-mouth pipette into 600 mL polycarbonate bottles (one copepod per bottle). *Calanus finmarchicus* females had an average prosome length (PL) of 2.7 ± 0.1 mm and *C. glacialis* females had an average PL of 3.4 ± 0.4 mm.

Ten females of each species were incubated in the natural phytoplankton community (200 μg C L^{-1}) spiked with the toxic diatom *P. seriata*, at concentrations of around 200 μg C L^{-1}. As a control, 10 females of each species were incubated separately in polycarbonate bottles containing only the natural phytoplankton community (around 400 μg C L^{-1}, pH 7.88, Salinity = 35). The treatment with *C. glacialis* and *C. finmarchicus* including the toxic diatom *P. seriata* are afterwards referred to as GT and FT, respectively, and the non-toxic treatments are accordingly referred to as G and F (Table II).

The copepods were incubated for 10 days under a 12 h light:12 h dark cycle at 9 ± 3 μmol photons m^{-2} s^{-1} (Osram L, 36 W/840, Lumilux Cold White light tubes) and under *in situ* temperature (4 ± 1.5°C). The light intensity resembled the light conditions at 20 m depth, the origin of the phytoplankton community.

Eggs and FPs were harvested daily by sieving the bottle content through a 100-μm filter. The copepods were gently taken from the submerged filter by a wide-mouth pipette and transferred into an ice-chilled six-chamber Multiwell containing filtered sea water. Separately for each of the four treatments (F, G, FT and GT), the water from the replicate flasks was subsequently pooled in 10 L polycarbonate containers and gently rotated to facilitate mixing. In order to provide the copepods with saturated food supply (>400 μg C L^{-1}), every day 1 mL of the pooled phytoplankton solution of each of the four treatments was sampled and fixed with 1% (final concentration) of Lugol's solution and the cell concentration quantified using a Sedgewick Rafter counting chamber under an inverted microscope (Olympus CKX3). At least 400 cells were counted. If the calculated carbon concentration was below 400 μg C L^{-1}, phytoplankton was added to achieve saturating conditions. Thereafter, the water was poured back into the original 600 mL polycarbonate bottles and each copepod was returned into a bottle with a wide-mouth pipette. Every 12 h each experimental bottle was gently turned around its own axis to keep the phytoplankton in suspension.

Table II: Abbreviations of the non-toxic and toxic treatments of C. finmarchicus *and* C. glacialis *and the respective sample size (number of individuals)*

Species and treatment	Abbreviation	Sample size
C. finmarchicus in non-toxic treatment	F	9
C. finmarchicus in toxic treatment[a]	FT	8
C. glacialis in non-toxic treatment	G	7
C. glacialis in toxic treatment[a]	GT	5

[a]Toxic treatments contain *P. seriata* besides the natural phytoplankton community.

For the hatching experiment, all eggs produced by one female were quantified and mouth pipetted into one chamber of a six-chamber Multiwell filled with 0.45 μm filtered sea water on 6 days of the experiment (i.e. Days 1, 3, 5, 7, 8, 9). The eggs were incubated for at least 4 days to allow all viable eggs to hatch (Jung-Madsen and Nielsen, 2015), under the same experimental conditions as described before but at a slightly lower irradiance (ca. 5 μmol photons m^{-2} s^{-1}). After 4 days, the samples were fixed with a drop of Lugol's solution and the number of hatched nauplii and intact eggs were counted and the individual hatching success calculated as the amount of nauplii hatched per female divided by the total number of eggs laid per female. The combined effect of hatching success and egg production was evaluated by calculating the reproductive output (Nørregaard *et al.*, 2014), by multiplying the average hatching success with the average amount of eggs produced per female. On days where the eggs were not used in the hatching experiment (Days 2, 6 and 10), they were quantified and their DA content analysed.

Every day, the FP of each copepod were washed off from the filter into a 6-chamber Multiwell, where they were counted under a stereo microscope (Leica Wild M3C; 10× magnification). The FP of the replicate bottles of each treatment were pooled and the length and width of 10 randomly selected FP were measured under a light microscope (Leitz Labovert FS; 10× magnification). The FP volume was calculated according to Swalethorp *et al.* (Swalethorp *et al.*, 2011), assuming a cylindrical shape, i.e. $V = L \times \pi \times (W/2)^2$. The total volume of the daily FP production was calculated as the total number of FP on a given day multiplied by the average FP volume of the treatment for that day. On Day 2, 4 and 6 of the experiment the DA content of the FP was analysed.

At the end of the experiment (i.e. Day 10), the PL of all copepods used in the experiment ($n = 29$, Table II) was measured under a light microscope (Leitz Labovert FS). The copepods' C content (mg C) was calculated from its PL (in mm) with the following regression equation: $0.0018 \times PL^{4.10}$ (Swalethorp *et al.*, 2011; according to a regression equation from pre/early bloom condition).

Domoic acid analyses

Samples for DA analyses were filtered through a GF/F (Whatman) filter using moderate vacuum and stored in plastic vials at −20°C. For measuring DA content of the samples, toxin extraction and subsequent liquid chromatography coupled with tandem mass spectrometry were performed as described in Tammilehto *et al.* (Tammilehto *et al.*, 2012). The detection limit of DA was

6.82 ng per sample. Results below this concentration were defined as not determined (nd).

In order to compare the amount of DA found in eggs, FP and the copepods, the measured DA concentrations were transformed to carbon-specific concentrations, i.e. ng DA μgC^{-1}. From the FP volume V_{FP} (μm^3) the FP weight in μg C could be calculated for *C. finmarchicus* and *C. glacialis* with the following regression equation from Swalethorp *et al.* (Swalethorp *et al.*, 2011): $V_{FP} \times 4.30 \times 10^{-8}$. From measurements of the egg volume V_{egg} (μm^3), the egg weight in μg C was calculated by: $V_{egg} \times 1.10 \times 10^{-7}$ (Swalethorp *et al.*, 2011).

Additionally, the daily rate of DA excretion via FP and eggs was calculated. Based on the DA concentration found in eggs and FP at the end of the experiment (Day 10) and the average daily egg and FP production per female over the 10-day experimental period, the daily DA excretion via FP and eggs was calculated. The DA concentration found at Day 10 was used for the calculation, assuming that the copepods feeding conditions were in equilibrium at Day 10. It has to be noted, that the copepods were not allowed time to clear their guts before toxins were measured, which could have led to a slight overestimation of DA found within their body.

Data analysis

The statistical analysis of the data was done by using R (R Core Team, 2015, version 3.1.3). A non-parametric bootstrap with 100 000 iterations was used to analyse the null hypothesis that there is no difference in the hatching success of the copepods in the toxic and non-toxic treatment, i.e. between F and FT, as well as G and GT.

The same method was applied to the FP production and volume with the null hypothesis that there is no difference in the FP production or volume between the toxic and the non-toxic treatment for each species. Moreover, this method was used to analyse the null hypothesis that there is no difference in the cellular concentration of *P. seriata* in the toxic treatment of *C. finmarchicus* and *C. glacialis*. Additionally a permutation test with 10 000 iterations was applied to analyse whether the carbon concentration of the phytoplankton solution had an effect on the egg production, hatching success and FP production. Differences were considered statistically significant when $P < 0.05$.

To analyse whether there was a difference between the numbers of eggs produced in the toxic and the non-toxic treatment of each species, a zero-inflated, negative binomial distribution with log-link was applied using the R package glmmADMB (Fournier *et al.*, 2012). A Pearson's χ^2 test using the pscl package in R was applied to analyse whether the probability of copepods laying zero eggs in the toxic and non-toxic treatment was equal. All mean values stated in the text are given as the mean $\pm$ standard deviation.

RESULTS

Phytoplankton

The copepods experienced saturated food concentrations (435 ± 99 μg C L^{-1}) throughout the experiment. Carbon concentrations did not influence any of the rates measured, i.e. FP production, egg production and hatching success. Therefore, we can assume that any effect on the measured variables was solely caused by the presence/absence of DA in the copepod's diet.

The dominant small-celled phytoplankton were primarily *Phaeocystis*, mainly *P. pouchetii*, and in addition the diatoms *Leptocylindrus minimus*, *Chaetoceros minimus* and *Chaetoceros gelidus* were abundant. Especially the large-celled phytoplankton, varied considerably in size and thus volume owing to its high species diversity. The chain-forming diatom *Thalassiosira* spp. was the prevailing large phytoplankton genus and the diatoms *Navicula* spp. (*N. pelagica*, *N. directa* and *N. distans*), as well as *Fragilariopsis* spp. (mainly *F. cylindrus*) were frequently encountered. The diatom species *Pleurosigma normanii* was also found sporadically. Identifications were based on Throndsen *et al.* (Throndsen *et al.*, 2007) and Chamnansinp *et al.* (Chamnansinp *et al.*, 2013). The dominant phytoplankton species in the spring bloom are typical diatom spring-bloom species of Disko Bay (Levinsen *et al.*, 2000).

The DA content of the phytoplankton suspensions in the toxic treatments containing *C. finmarchicus* (FT) and *C. glacialis* (GT) were similar ($t = 0.1844$, $df = 42.272$, $P = 0.8546$; Table III). Likewise, there was no significant difference in the cell concentration of *P. seriata* in the two treatments ($P = 0.2077$), with an average of 769 ± 355 cells mL^{-1} in FT and 944 ± 205 cells mL^{-1} in GT.

The intracellular DA content of *P. seriata* increased over time with an average of 23–24% per day in FT and GT, respectively (Table III). In FT, the increase in *P. seriata*'s intracellular DA content was most pronounced in the second half with a 2.8-fold increase from Day 6 to 10, while *P. seriata*'s intracellular DA content increase was most prominent in the first half of the experiment in GT with a 2.6-fold increase from Day 2 to 6 (Table III).

Faecal pellet production

The FP production of both species was not affected by the DA content, size or composition of the phytoplankton community or *P. seriata* concentration.

Feeding on the DA-producing *P. seriata* did not affect the FP production. When comparing the toxic and non-

Table III: Summary of the domoic acid (DA) analysis [standard deviation (±) indicated for DA phytoplankton solution; 3 replicates]

DA concentration of/in	Treatment	Day 2	Day 6	Day 10
Phytoplankton solution (ng DA 10^{-2} mL^{-1})	F	nd	nd	nd
	FT	24.98 ± 4.09	70.48 ± 1.34	210.19 ± 22.15
	G	0.00	nd	0.00
	GT	28.46 ± 3.01	98.17 ± 7.46	170.78 ± 8.76[a]
P. seriata cell (pg DA $cell^{-1}$)	FT	0.46	0.83	2.34
	GT	0.36	0.93	1.98[a]
Eggs (ng DA egg^{-1})	F	nd	nd	nd
	FT	nd	0.04	0.07
	G	nd	nd	nd
	GT	nd	0.11	0.06
Total FP volume (ng DA mm^{-3})	F	nd	nd	nd
	FT	nd	167.79[a]	221.14
	G	nd	nd	nd
	GT	nd	173.68	89.59
FP (ng DA $pellet^{-1}$)	F	nd	nd	nd
	FT	nd	0.71	1.07
	G	nd	nd	nd
	GT	nd	0.92	0.60

Values below the detection limit are indicated by nd.
[a]A standard error of 5%; while all other concentrations have a standard error of 9.75% with respect to the toxin analysis' accuracy. Note that data for FT on Day 10 is based on the cellular phytoplankton concentration of the previous day due to missing data.

toxic treatments, there was no significant difference in the daily FP production of *C. finmarchicus* ($P = 0.3662$) and *C. glacialis* ($P = 0.8283$), respectively. Additionally there was no significant difference in the FP volume in the toxic and non-toxic treatment of *C. finmarchicus* ($P = 0.2564$) and *C. glacialis* ($P = 0.1175$).

The median FP production rate of *C. finmarchicus* during the 10-day experimental period ranged between 60 ± 34 pellets $female^{-1}$ d^{-1} in the non-toxic and 62 ± 34 pellets $female^{-1}$ d^{-1} in the toxic treatments (Fig. 2A). The average FP volume ranged between 4.3 ± 0.9 × 10^6 μm^3 in the non-toxic and 3.62 ± 0.62 × 10^6 μm^3 in the toxic treatments. The median FP production rate of *C. glacialis* was 52 ± 27 pellets $female^{-1}$ d^{-1} in the non-toxic and 52 ± 25 pellets $female^{-1}$ d^{-1} in the toxic treatment (Fig. 2A). *Calanus glacialis*' average FP volume was 5.93 ± 0.91 × 10^6 μm^3 in the non-toxic and 5.44 ± 1.16 × 10^6 μm^3 in the toxic treatments.

FP of *C. finmarchicus* contained slightly more DA than those of *C. glacialis* with an average of 194 ng DA mm^{-3} and 0.86 ng DA $pellet^{-1}$ in FT compared with 132 ng DA mm^{-3} and 0.76 ng DA $pellet^{-1}$ in GT. While there was an increase of DA in the FP in FT from Day 6 to Day 10, it decreased in the GT treatment (Tables III and IV).

Egg production and hatching

Feeding on DA-containing *P. seriata* affected neither copepod egg production nor hatching success. The egg production in the toxic and non-toxic treatment was not significantly different in *C. glacialis* ($P = 0.73$, zero-inflation = 1.08, log-likelihood = −440.20). In *C. finmarchicus* there was no significant difference either, although there was a weak indication that the egg production was affected by the different treatments ($P = 0.061$, zero-inflation = 0.556, log-likelihood = −463.68), probably attributed to an unusually high egg production of FT at Days 6 and 10 (Fig. 2B). *Calanus finmarchicus* females laid on average 11 ± 17 and 15 ± 25 eggs d^{-1} with a maximum daily egg production of 65 and 113 eggs d^{-1} in the non-toxic and toxic treatments, respectively (Fig. 3).

Calanus glacialis had an average egg production of 19 ± 24 eggs d^{-1} in the non-toxic treatment and 20 ± 24 eggs d^{-1} in the toxic treatment (Fig. 3). The maximum daily egg production ranged between 79 and 69 eggs d^{-1} in the non-toxic and toxic treatments, respectively (Fig. 3). Overall *C. glacialis* had a higher cumulative egg production per female than *C. finmarchicus*.

The probability of copepods laying zero eggs in the toxic and non-toxic treatments was not significantly different for *C. finmarchicus* ($\chi^2 = 0.2036$, $df = 1$, $P = 0.6518$) nor for *C. glacialis* ($X^2 = 0$, df = 1, $P = 1$).

No correlation was found between egg production and the carbon content of the phytoplankton suspension, cell concentration of large phytoplankton cells or *P. seriata* concentration. Moreover, the number of FP produced did not correlate with egg production in any of the treatments. We can therefore conclude that the egg production of *C. glacialis* and *C. finmarchicus* is not affected by feeding on DA containing phytoplankton.

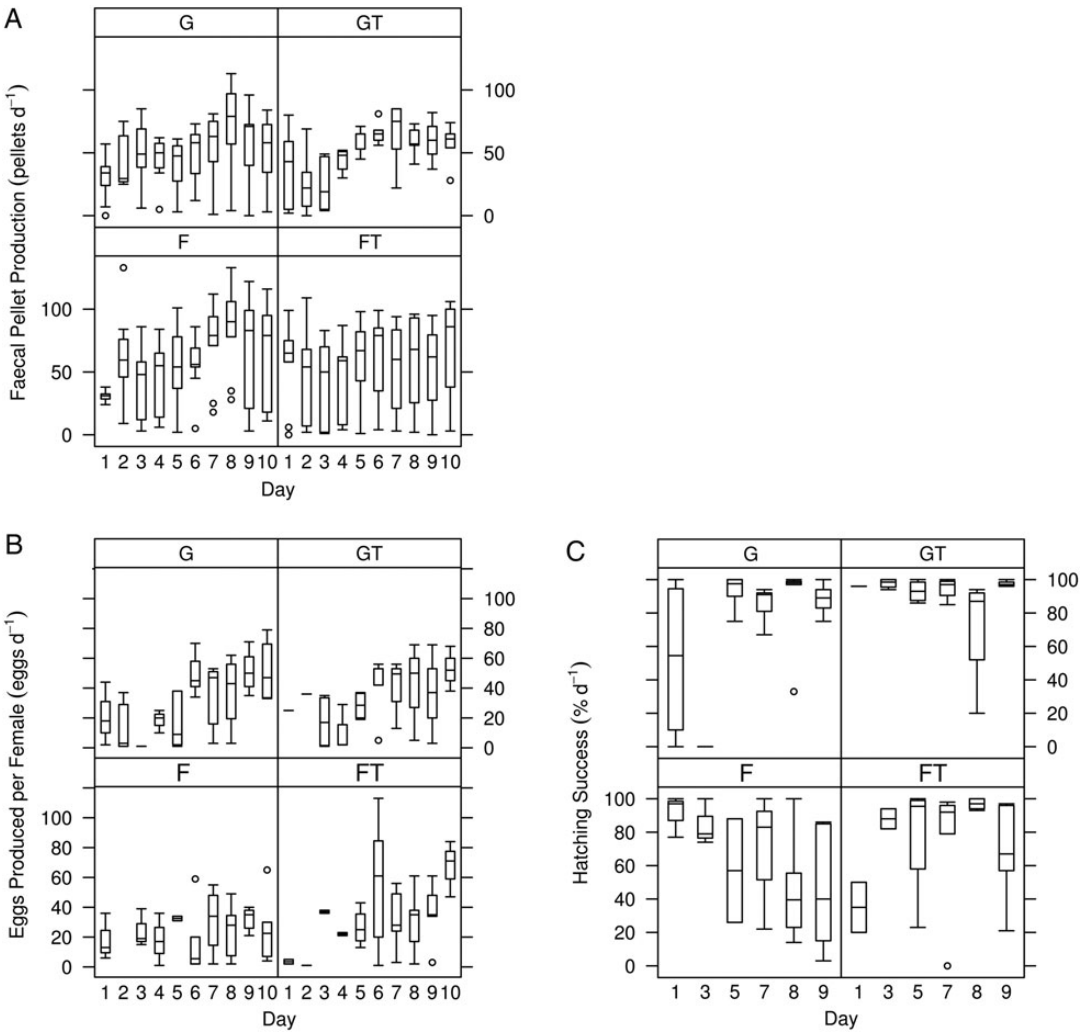

Fig. 2. (**A**) Faecal pellet production (number of faecal pellets produced per female); (**B**) number of eggs produced per female during the course of the experiment (data from females laying no eggs on a particular day were excluded) and (**C**) hatching success of eggs in the non-toxic and toxic treatments of *C. finmarchicus* (F and FT) and *C. glacialis* (G and GT), respectively. The bar in the middle of the box represents the median and outliers are represented by dots.

Table IV: Carbon-specific domoic acid (DA) concentration (ng DA μg^{-1} C^{-1}) of the phytoplankton solution, faecal pellets, eggs and C. finmarchicus *and* C. glacialis *females*

Domoic acid (DA) concentration (ng DA μg^{-1} C^{-1}) of	*C. finmarchicus*			*C. glacialis*		
	Day 2	Day 6	Day 10	Day 2	Day 6	Day 10
Phytoplankton solution	0.60	1.38	4.35	0.58	1.68	4.19
Faecal pellets	nd	0.22	0.42	nd	0.39	0.32
Eggs	nd	0.01	0.01	nd	0.04	0.02
Female	NA	NA	0.01	NA	NA	0.08
Total			0.44			0.42

Values below the detection limit are indicated by nd and missing data by NA.

There was no significant difference in the hatching success of the toxic and non-toxic treatments of *C. glacialis* ($P = 0.1743$) and *C. finmarchicus* ($P = 0.0624$). *Calanus. finmarchicus* had an average hatching success of 60 ± 32% and 77 ± 31% in the non-toxic and toxic treatments, respectively, during the 10-day experimental period ($n = 6$; Fig. 3) and in 5 out of 6 days, the median hatching success was greater in the toxic treatment, than in the non-toxic one for *C. finmarchicus* (Fig. 2C). The hatching success of *C. glacialis* was statistically more similar with a mean hatching success of 81 ± 30% in the non-toxic treatment and 91 ± 17% in the toxic treatment (Fig. 3) while the median hatching success was only higher on 2 out of 5 days (Fig. 2C). The hatching success of GT was

higher than in FT ($P = 0.077$). As a result of a higher egg production and hatching success of *C. glacialis*, the cumulative reproductive output was higher than that of *C. finmarchicus*. Another difference between the species was that the hatching successes of *C. finmarchicus* varied to a great extent in both treatments while *C. glacialis* showed more constant hatching rates with a generally lower standard deviation, in particularly in the toxic treatment (Fig. 3).

Fate of domoic acid

At the end of the experiment, DA concentrations reached 0.91 ng DA per copepod in FT and 20.98 ng DA per copepod in GT. When considering the size and weight difference of the two copepods, *C. glacialis* had a 8-times-higher carbon-specific DA accumulation with 0.08 ng DA μgC^{-1} compared with 0.01 ng DA μgC^{-1} in *C. finmarchicus* (Table IV).

In both species, more DA was excreted through the FP compared with the eggs (Fig. 4). The daily rate of DA excretion via the FP was greater in *C. finmarchicus* compared with *C. glacialis*, while *C. glacialis* showed a greater excretion of DA via the eggs compared with *C. finmarchicus* (Fig. 4).

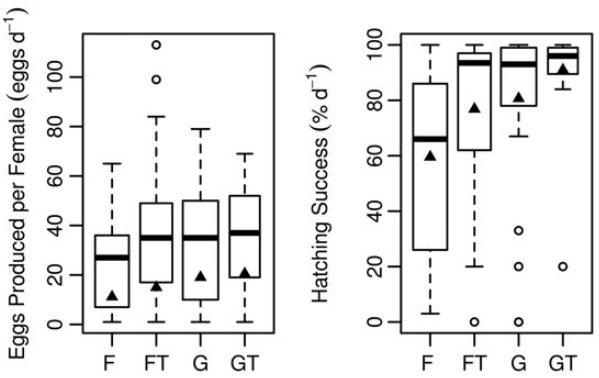

Fig. 3. Boxplot of the number of eggs produced per female per day (left) and the hatching success of eggs (right) in the non-toxic and toxic treatments of *C. finmarchicus* (F and FT) and *C. glacialis* (G and GT), respectively. The thick bar in the middle of the box indicates the median, the black triangle indicates the mean and outliers are represented by dots. The boxes are drawn with widths proportional to the square-roots of the number of observations in the groups. Note that days where the females did not produce any eggs were excluded from the egg production.

DISCUSSION

Feeding on DA-producing *P. seriata* did not affect any of the variables measured, i.e. FP production, egg production or hatching success, despite increasing DA concentrations of the grazed *P. seriata* during the experiment.

Domoic acid content of phytoplankton

The intracellular DA content of *P. seriata* increased over time with an average of 23% per day in both toxic treatments (Table III). This agrees with studies that used female *Calanus* or *Calanus* copepodites grazing on *P. seriata* and resulted in an increase in *P. seriata* toxin content (Harðardóttir *et al.*, 2015; Tammilehto *et al.*, 2015). These studies also suggest that the increase in DA may have been induced by chemical cues, since it could not be explained by changes in nutrient concentrations or pH (Harðardóttir *et al.*, 2015; Tammilehto *et al.*, 2015). DA production in *P. seriata* can be influenced by a range of different factors, nevertheless the purpose of DA production in *P. seriata* is still unresolved (Lelong *et al.*, 2012). The hypothesis that DA acts as a grazer-deterrent has been

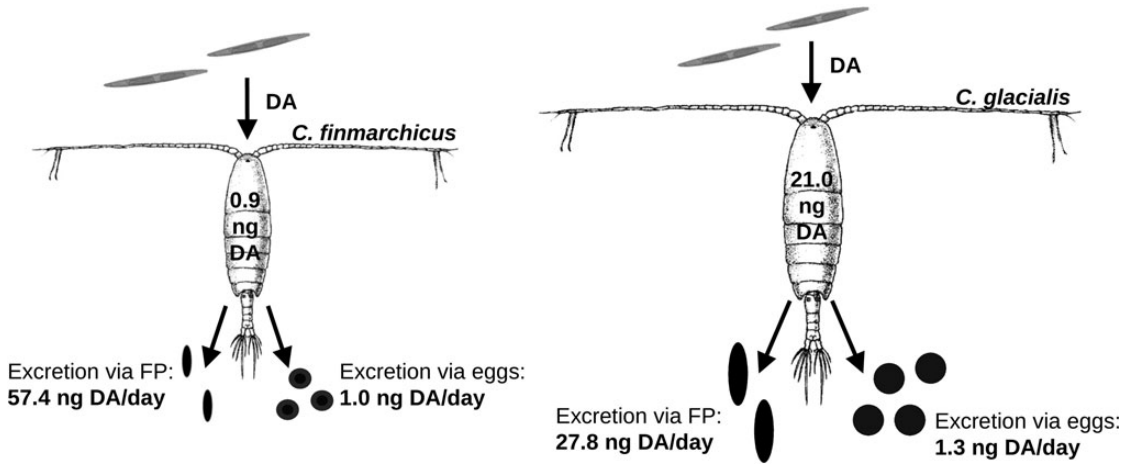

Fig. 4. Amount of domoic acid (DA) found per female at the end of the experiment (Day 10) in *C. finmarchicus* (left) and *C. glacialis* females (right) and the rates of DA excretion (ng DA day^{-1}) through their eggs and faecal pellets (FP). The rates are based on the DA concentration in egg and FP at Day 10 and the average egg and FP production per female throughout the experiment.

rejected in some studies (Tammilehto *et al.*, 2012; references within Lelong *et al.*, 2012).

Faecal pellet production

The FP volume and number of FP produced were unrelated to the phytoplankton biomass and composition, illustrating that food conditions were indeed saturated throughout the experiment. Feeding on *P. seriata* did not affect the number of FP produced nor the FP volume in any of the species. This confirms the result of Tammilehto *et al.* (Tammilehto *et al.*, 2012) who found no difference between FP production of the same species when feeding on unialgal cultures of toxic *P. seriata* and the non-toxic *P. delicatissima*. Accordingly grazing was not affected by DA.

FP of *C. finmarchicus* contained more DA at Day 10 compared with *C. glacialis,* while the opposite was true for Day 6 (Tables III and IV). This is very likely attributed to the differences in the intracellular DA concentration of *P. seriata* which was higher in FT on Day 10 while it was higher for GT on Day 6 (Table III). Moreover, this indicates that ingested DA is directly excreted via FP. This is consistent with the hypothesis that copepod FP production can be directly related to ingestion within a few hours (see, e.g. Besiktepe and Dam, 2002). Therefore, FP production can be used as a proxy of grazing, as in this study.

Effect of feeding on *p. seriata* on egg production and hatching

Feeding on *P. seriata* and the subsequent accumulation of DA had neither an effect on the egg production rate nor on the share of females producing eggs. Moreover, there was no significant difference in the probability of copepods laying no eggs in the two treatments. Although no significant difference was found, the mean egg production was slightly higher in the toxic treatment of both species (Fig. 3).

The average daily egg production of *C. finmarchicus* in this study was higher compared with *in situ* rates reported from the same region during a phytoplankton bloom, while the average egg production rate of *C. glacialis* was between reported rates (Swalethorp *et al.*, 2011; Kjellerup *et al.*, 2012). Other studies from the same region reported a slightly higher egg production for *C. finmarchicus* (Madsen *et al.*, 2001; Niehoff *et al.*, 2002). The maximum daily egg production rate of *C. glacialis* exceeded the values reported by Niehoff *et al.* (Niehoff *et al.*, 2002) while it was slightly lower than in the study of Swalethorp *et al.* (Swalethorp *et al.*, 2011).

There was an overall increase in egg production in both species during the second half of the experiment (Fig. 2B). Likewise in a study on *C. glacialis* and *C. finmarchicus* sampled during a bloom from the same region and kept under similar experimental conditions, i.e. under food saturated conditions at 5°C, egg production showed an initial lag phase of 4 days (Kjellerup *et al.*, 2012).

Mean hatching success was not significantly different between the toxic and the non-toxic treatments (Fig. 3), although like egg production, the average hatching success was higher in the toxic treatment for both species. In studies with similar experimental conditions but without *P. seriata* as food, *C. finmarchicus* had a slightly higher average hatching success with 88 ± 6% (Jung-Madsen and Nielsen, 2015) and 91 ± 13% (Campbell and Head, 2000), while reported hatching rates of *C. glacialis* were slightly lower under similar conditions with 78 ± 7% (Jung-Madsen and Nielsen, 2015). Considering that hatching success of *C. finmarchicus* and *C. glacialis* also remains unaltered by toxins such as the polycyclic aromatic hydrocarbon pyrene (Grenvald *et al.*, 2013), the fecundity of these species seems to be quite resilient to a variety of toxins

Overall there was no effect of DA on the fecundity of *C. finmarchicus* and *C. glacialis*. Similarly, studies conducted with *Acartia tonsa* and *Temora longicornis* (Lincoln *et al.*, 2001) as well as *Acartia clausi* (Maneiro *et al.*, 2005) feeding on DA-containing phytoplankton did not show any effect on these species' fecundity. These copepods might have adapted to DA from *Pseudo-nitzschia* due to co-evolution, i.e. that during interaction with the potential toxic spp. through generations they have evolved a tolerance to DA-producing diatoms in Disko Bay.

Fate of domoic acid

Feeding on *P. seriata* resulted in accumulation of DA in the copepod eggs, FP and their body (Fig. 4). The DA concentrations per copepod reported by Tammilehto *et al.* (Tammilehto *et al.*, 2012) were higher with 35 ± 13 and 55 ± 10 ng DA copepod^{-1} in *C. finmarchicus* and *C. glacialis,* respectively, compared with this study (Fig. 4). While intracellular concentration of DA in *P. seriata* for *C. glacialis* was comparable in both studies, it was lower for *C. finmarchicus* in Tammilehto *et al.* (Tammilehto *et al.*, 2012) with 0.54 pg DA cell^{-1}, but the percentage of toxic cells in the phytoplankton differed. This may partly explain the low accumulation of DA in FT in the present study.

DA was excreted mainly through the copepods FP while excretion via the eggs was comparatively low (Fig. 4). In particular *C. finmarchicus*' daily rate of DA excretion via the FP was approximately 60 times higher compared with the amount of DA excreted through its eggs and the amount of DA retained in its body at the end of the experimental period (Fig. 4). The rate of DA

excretion via the FP in *C. glacialis* was ~50% lower than in *C. finmarchicus*, but still exceeded the DA concentration retained in its body and excreted via eggs. This indicates that the greatest share of DA that is taken up by the copepods when feeding on DA-producing phytoplankton and is removed from the body by means of defecation.

It has to be pointed out that the toxin concentration of the FP in this study might be underestimated due to DA leaking out of FP into the surrounding water as suggested by Tammilehto *et al.* (Tammilehto *et al.*, 2012). Consequently, the amount of DA measured in the copepods and their eggs might be overestimated.

Since DA is hydrophilic, it has been hypothesized that it does not bioaccumulate in aquatic organisms, but can concentrate in their digestive systems (see references within Trainer *et al.*, 2012). In *C. finmarchicus* the DA concentration of 0.91 ng DA per female was likely caused by yet unexcreted FP, since the DA concentration within one FP exceeded the DA concentration within the copepods (1.07 ng DA pellet^{-1}, Table III). Nevertheless, the study of Tammilehto *et al.* (Tammilehto *et al.*, 2012) recorded higher DA retention in *C. finmarchicus* even after 12 h starvation during which the gut will have been emptied and therefore concluded that *C. finmarchicus* assimilated DA in its tissue.

The accumulation of 20.98 ng DA per female in *C. glacialis* is above what the presence of a FP inside the copepod could explain (Table III). Even considering the possible underestimation of DA in FP, the DA accumulation of *C. glacialis* is still unlikely to result from a FP within its digestive tract, in view of the fact that the DA concentration in GT was 40 times higher that of its FP. One can thus assume that *C. glacialis* accumulates DA in its body tissue. Even though calanoid copepods grazing on *Pseudo-nitzschia* species have been reported to accumulate DA in their tissue (Lincoln *et al.*, 2001; Tammilehto *et al.*, 2012) it is surprising that *C. glacialis* accumulated more DA than *C. finmarchicus*, in view of the fact that DA is a lipophobic substance (Falk *et al.*, 1991; Lane *et al.*, 2010) and *C. glacialis* contains a greater amount of lipids than *C. finmarchicus* [*C. glacialis* dry mass (DM) is composed of 70% lipids compared with 31% lipids in *C. finmarchicus* DM (Falk-Petersen *et al.*, 2009)]. Alternatively, even though the cellular concentration of *P. seriata* was not significantly higher in GT compared with FT, the slight difference might have caused the greater accumulation of DA in *C. glacialis*.

Eggs of *C. glacialis* contained more carbon-specific DA than those of *C. finmarchicus* (Table IV). Beside the incorporation of DA during ovulation, it is possible, that DA adhered to the eggs' outer membrane, which is composed of a multi-layered eggshell. The egg membrane of *C. glacialis* has spines, while *C. finmarchicus*' eggs have a smooth surface (Nielsen *et al.*, 2014). Accordingly, the surface area of *C. glacialis*' egg membrane is greater, providing a larger surface for DA molecules to attach to, which could explain the greater relative DA concentrations in eggs of *C. glacialis* females.

CONCLUSION

Overall, feeding on the DA-producing *P. seriata* did not affect the fecundity or the FP production of *C. finmarchicus* and *C. glacialis*. The greatest fraction of DA measured was excreted via FPs in both species, while DA excretion via eggs was greater in *C. glacialis* compared with *C. finmarchicus*. Nonetheless, the two copepod species accumulated DA, with *C. glacialis* assimilating substantially more DA in its body compared with *C. finmarchicus*. Accordingly *C. glacialis* could efficiently transfer DA up the food chain where it has been shown to bioaccumulate, e.g. in whales (Leandro *et al.*, 2010; Doucette *et al.*, 2012). It would be interesting to investigate whether there is an effect on the survival and development time of nauplii when the maternal diet consisted of DA-producing phytoplankton. Future studies will have to resolve that issue.

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