Dietary protein requirement for somatic growth and gonad production in the sea urchin Strongylocentrotus intermedius at different life stages

Dietary protein requirement for somatic growth and gonad production in the sea urchin *Strongylocentrotus intermedius* at different life stages

Satomi Takagi[a]*, Natsuki Hasegawa[a], Tsuyoshi Watanabe[a], Yuichi Sakai[b], Tatsuya Unuma[a,c]

[a]Kushiro Field Station, Fisheries Resources Institute, Japan Fisheries Research and Education Agency, Kushiro, Hokkaido 085-0802, Japan
[b]Hakodate Fisheries Research Institute, Hokkaido Research Organization, Hakodate, Hokkaido 040-0051, Japan
[c]Present address: Graduate School of Agricultural Science, Tohoku University, Sendai, Miyagi 980-8572, Japan

*Corresponding author
Satomi Takagi
Email: takagi_satomi02@fra.go.jp

Abstract

The rise in the demand for sea urchin aquaculture requires the optimization of dietary protein levels to maximize somatic growth and gonad production throughout the aquaculture process. This study aimed to investigate the dietary protein requirement for somatic growth and gonad production of the sea urchin *Strongylocentrotus intermedius* at different life stages considering protein leaching from diets during seawater immersion. Feeding trials were performed on three sizes of *S. intermedius*: S, small urchins prior to gonad differentiation; M, medium urchins following gonad differentiation; and L, large

urchins close to the biological minimum size. Two feeding trials using diets with different gluten (protein source) levels (experiment I; 5, 10, 15, 20, 30, and 40%: experiment II; 0, 2, 4, 7, and 10%) were conducted for each size group. The diet ingredients were selected to minimize protein leaching, and the protein content of each diet was determined from the average content after immersion in seawater for 24 and 72 h. No significant differences were observed in the specific growth rate (SGR) of body weight in experiment I in all size groups. The SGRs of the body weight of urchins fed 4 and 7% gluten diets were significantly higher than those fed diets containing 0% gluten in experiments SII and MII, and a similar tendency was observed for SGRs for the body weight and test diameter of large urchins. Gonad indices of sea urchins increased with an increase in dietary protein content in experiments MII and LII, and the gonad indices of urchins fed 15 and 20% gluten diets were significantly higher than those fed 5% gluten diet in experiments MI and LI. Broken-line regression analyses on urchins in experiment II estimated the protein requirement for somatic growth to be 2% regardless of the urchin size, which is well below the range of feed protein contents (9–50%) used in previous studies. The analyses on urchins in experiment I estimated the protein requirement for gonad production of medium and large urchins to be 13% and 15%, respectively, which was lower than that previously reported (>20%). These results indicate that previous

45 studies overestimated the protein requirement owing to protein leaching during the
46 immersion of feed in seawater. Our findings indicate that protein content in sea urchin
47 feed diets can be considerably reduced irrespective of their cultivation stage from seed to
48 market size.
49 Keywords: body weight; gonad index; optimum protein level; protein leaching; test
50 diameter
51

52 1. Introduction

53 Global fish and shellfish consumption has increased over the past few decades (FAO,
54 2022a; 2022b). Sea urchin gonad is a premium shellfish delicacy with high commercial
55 value, and it is consumed in Japan, China, Korea, the United States, Canada, Chile, and a
56 few other European countries (Sun and Chiang, 2015). The Tokyo Metropolitan Central
57 Wholesale Market, Japan—the largest wholesale sea urchin market in the world (Sun and
58 Chiang, 2015)—witnessed a steep rise in the yearly average price of sea urchin gonads
59 from 6,625 JPY kg^{-1} in 2011 to 20,502 JPY kg^{-1} in 2021 (Metropolitan Central Wholesale
60 Market, 2022). Additionally, the commencement of the import of live urchins, fresh
61 gonads, and frozen gonads into Europe and Oceania since 2012 (FAO, 2022c) indicates a
62 rise in their global demand. However, world sea urchin landings have stagnated (FAO,

63 2022d) owing to stock depletions caused by overfishing since the 1990s (Andrew, 2002).
64 Thus, the production of hatchery-raised urchins in aquaculture systems was proposed to
65 meet market demands (McBride, 2005; Pearce, 2010; Walker et al., 2015). The cultivation
66 of sea urchins from egg to market size takes over three years, requiring a constant supply
67 of feed throughout the year; fresh *Laminariales* kelp is commonly used as feed for sea
68 urchin aquaculture (Unuma and Kayaba, 2015; Lawrence et al., 2019), and its availability
69 varies depending on the year, season, and location. Therefore, artificial feeds should be
70 developed to stabilize the supply and nutritional value of sea urchin feeds without
71 seasonal and locational limitations.

72 To develop sea urchin feeds, it is necessary to clarify the requirement of various
73 nutrients in diets. Sea urchin aquaculture includes cultivation from egg until the market
74 size, as well as the enhancement of gonads. Sea urchin body growth initially occurs after
75 metamorphosis; both body and gonadal growths occur after the gonads differentiate.
76 Studies on wild populations of *Strongylocentrotus droebachiensis* (Minor and Scheibling,
77 1997; Meidel and Scheibling, 1999) and *Hemicentrotus pulcherrimus* (Agatsuma and
78 Nakata, 2004) suggest that body and gonad growth may not occur independently of each
79 other. However, the optimum nutrient level required for the growth of each body site is
80 not necessarily the same, as chemical compositions vary among the body sites (Fuji, 1967;

Hammer et al., 2006a). Generally, the nutrients and energy allocated toward somatic growth decrease, and those allocated toward gonad production increase with increasing body size, i.e., aging (e.g., Fuji, 1967; Fernandez and Boudouresque, 2002). Moreover, with the increase in body size, the energy allocated toward maintenance increases, whereas the assimilation efficiency decreases (Fuji, 1967; Fernandez and Boudouresque, 2002). These observations suggest that the nutrient requirements of sea urchins could vary based on their life stage and body site.

Protein is one of the most essential nutrients in sea urchin diets. Proteins play a crucial role in many biological processes, including reproduction, growth, and maintenance of body tissue (Watts et al., 2020). Previous studies suggest that the protein requirements for somatic growth of young *Lytechinus variegatus* (approximately 4 g body weight, BW) is >30% (Heflin et al., 2016), and those of juvenile *Strongylocentrotus intermedius* (1.53 g BW; Zuo et al., 2017), young *P. depressus* (approximately1.6 g BW; Akiyama et al., 2001) and adult *L. variegatus* (23 g BW; Hammer et al., 2006b) are approximately 20%. The protein requirements for gonad production in young *P. depressus* (Akiyama et al., 2001), *Paracentrtotus lividus* (approximately 17 g BW; Lourenço et al., 2020), young *L. variegatus* (Heflin et al., 2016), and juvenile *S. intermedius* (Zuo et al., 2017) are suggested to be ≥30%, and those of adult *L. variegatus* (Hammer et al., 2006b)

99 and adult *Strongylocentrotus droebachiensis* (>50mm test diameter, TD; de Jong-
100 Westman et al., 1995; Pearce et al., 2002) are 20%. However, the effect of life stage on
101 protein requirement has not been systematically studied in a particular species.
102 Additionally, a portion of the protein included in the diets of slow-feeding marine animals,
103 such as several gastropods, crustaceans, and echinoids, is lost as it leaches from their diets
104 into the water before consumption (Durazo-Beltrán and Viana, 2001; Dominy et al., 2003;
105 Argüello-Guevara and Molina-Poveda, 2013; Watts et al., 2020). Our previous study
106 demonstrated that >40% of the protein content of artificial diets can decease after
107 seawater immersion when the protein source included in artificial diets does not generate
108 a network structure and the binder concentration is low; hence, protein can leach from the
109 diet upon immersion in seawater (Takagi et al., 2022). Therefore, to estimate the optimum
110 dietary protein level for gonad production in adult *Mesocentrotus nudus*, we used two
111 novel approaches to eliminate the influence of protein leaching in seawater: (1) the
112 experimental diets were designed to reduce protein leaching to the extent possible during
113 seawater immersion; (2) the actual protein contents of the diets following immersion in
114 seawater were determined and used in the model analyses to determine the optimum
115 protein level (Takagi et al., 2022). The optimum level was estimated to be 12%, which
116 was much lower than those reported for other sea urchin species (20–35%; de Jong-

117 Westman et al., 1995; Akiyama et al., 2001; Pearce et al., 2002; Hammer et al., 2006b;
118 Heflin et al., 2016; Lourenço et al., 2020). Two scenarios are likely here: either previous
119 studies have overestimated the dietary protein requirement of sea urchins because of the
120 protein leaching, or the low protein requirement is specific for gonad production in adult
121 *M. nudus*.

122 *Strongylocentrotus intermedius* is commercially harvested in northern Japan, China,
123 and Russia. This species, together with *M. nudus*, accounts for over two-thirds of the total
124 sea urchin landed in Japan (Unuma, 2015). Wild *S. intermedius* requires 2 years to reach
125 the biological minimum size (30–35 mm TD; Fuji, 1967), whereas urchins under satiation
126 feeding conditions in the laboratory can mature at 1 year of age (reviewed by Agatsuma,
127 2020). The gonad index for commercially caught *S. intemedius* is considered to be ≥15
128 (Machiguchi et al., 2012). In Japan, the hatchery seed of *S. intermedius* has been produced
129 to reseed the native wild stock (Agatsuma, 2015). Market size *S. intermedius* (≥40 mm
130 TD), grown from artificial seeds, has been commercially produced in northern Japan and
131 China in regions with abundant availability of fresh *Saccharina* kelp (Liu and Chang,
132 2015; Unuma and Kayaba, 2015; Lawrence et al., 2019). Furthermore, the rise in seawater
133 temperature because of global warming would decrease the juvenile recruitment of wild
134 *S. intermedius* (Gouda et al., 2017; Gouda and Agatsuma, 2020) and reduce the

distribution of *Saccharina* kelp in northern Japan (Sudo et al., 2020). This would further expand the scope for *S. intermedius* aquaculture, and boost the production of suitable diets to replace kelp. Zuo et al. (2017) investigated the protein requirement for somatic growth and gonad production of juvenile *S. intermedius* (1.53 g body weight, BW) as mentioned above (the third paragraph in this section). However, to the best of our knowledge, the dietary protein requirement of juvenile *S. intermedius* before gonad differentiation, as well as that of adults of the same species, has not been investigated yet.

The aim of this study was to determine the dietary protein requirement for somatic growth and gonad production of *S. intermedius* at different life stages, eliminating the influence of protein leaching. Feeding trials were conducted using three different sizes of hatchery-reared sea urchins: (1) small juvenile sea urchin (approximately 6 mm TD; 0.1 g BW) at 0+ years of age before gonad differentiation, (2) juvenile sea urchin (approximately 13 mm TD; 1 g BW) at 0+ years of age after gonad differentiation, and (3) young sea urchin (approximately 30 mm TD; 12 g BW) at 1+ years of age and reaching the biological minimum size. The sea urchins were fed diets containing varying levels of proteins, designed to minimize protein leaching during seawater immersion (Takagi et al., 2022). The actual protein content of the diets after immersion into seawater was obtained and used to estimate the protein requirement for each body size based on the modeled

153 relationships between the dietary protein level and the resultant somatic growth and gonad
154 production (Takagi et al., 2022). The results of this study could provide practical solutions
155 for developing cost-effective sea urchin diets.
156
157 Materials and Methods
158 2.1. Sea urchin rearing
159 Four-month-old juvenile *S. intermedius* (about 3 mm TD), hatched and reared at a
160 hatchery in Akkeshi, Hokkaido, Japan, were transferred to the Kushiro Field Station of
161 the Japan Fisheries Research and Education Agency, Hokkaido (42°57'01" N, 144°26'35"
162 E) in July 2020 (Group A) and 2021 (Group B). The sea urchins were reared in sand-
163 filtered running seawater and mainly fed naturally occurring attached diatoms in a rearing
164 tank for one month; thereafter, the sea urchins were fed fresh (live) *Laminariales* kelp *ad*
165 *libitum* until they grew to a size suitable for use in the feeding experiments.
166
167 2.2. Diet preparation
168 The protein source (wheat gluten) and binder source and concentration (50% potato
169 starch) of the experimental diets were selected because of their high shape stability and
170 low protein leaching capacity (Takagi et al., 2022). Experimental diets containing

171 different levels of wheat gluten (Gluten, from wheat, FUJIFILM Wako Pure Chemical
172 Corporation, ≤100% purity) were prepared according to Takagi et al. (2022). Each diet
173 contained 50% potato starch (Starch, from Potato, FUJIFILM Wako Pure Chemical
174 Corporation, Osaka, Japan; ≤100% purity) and 10% powdered *Saccharina japoniva* var.
175 *diabolica* (collected from Akkeshi Bay, Hokkaido (43°01'17" N, 144°50'12" E) between
176 April and May 2021). Crystalline cellulose (FD-301, CEOLUS®, Asahi Kasei Corp.,
177 Tokyo, Japan; ≤100% purity) was used as the base material to balance the different gluten
178 concentrations (Table 1). Gluten and starch were of reagent grade, and cellulose was of
179 medicinal grade. The details of diet preparation are presented in Figure S1. One-and-a-
180 half times the amount of water was added to the dry ingredients. Before use in the feeding
181 experiments, a total of 50 g of each diet was dried at 60 °C for 72 h, and the crude protein,
182 crude lipid, ash, and carbohydrate contents in the diets (n = 2) were analyzed
183 (supplementary material S1). The proximate composition of each diet is shown in Table
184 1.
185
186 2.3. Feeding experiment
187 Six feeding experiments were conducted at the Kushiro Field Station using three
188 different size groups of *S. intermedius* at tenfold intervals of body weight; small urchins

189 before gonad differentiation (approximately 6 mm TD) were used for experiments SI and
190 SII, medium urchins with small five semi-transparent gonads visible to the naked eye
191 (Fuji, 1960) (approximately 13 mm TD) were used for experiments MI and MII, and large
192 urchins reaching the biological minimum size (approximately 30 mm TD) were used for
193 experiments LI and LII (Table 2). Sea urchins of each body size were subjected to two
194 feeding experiments involving different gluten concentrations in their diets: 5, 10, 15, 20,
195 30, and 40% gluten concentrations were used for experiments SI, MI, and LI, and 0, 2, 4,
196 7, and 10% gluten concentrations were used for experiments SII, MII, and LII (Table 1).
197 A gluten concentration of 10% was used in all experiments.
198
199 2.3.1. Experiment SI: small sea urchins fed with 5–40% gluten diets
200 Sea urchins were subjected to six feeding treatments with 5, 10, 15, 20, 30, and 40%
201 gluten diets (SI-5, SI-10, SI-15, SI-20, SI-30, and SI-40; Tables 1 and 3). The feeding
202 experiment was conducted from August 27 to September 15, 2021. At the start of the
203 experiment, 108 sea urchins (85–157 mg BW) from Group B were weighed using an
204 electronic balance. The sea urchins were divided into six treatments (18
205 individuals/treatment) (Table 3). The urchins in each treatment were housed in three
206 polyethylene baskets (L 8 cm × W 8 cm (bottom), L 10 cm × W 10 cm (upper) × H 11 cm,

207 with a 4 mm diameter open mesh on the sides higher than 1.3 cm from the bottom) at a
208 density of six individuals/basket (three baskets/treatment). The three baskets from each
209 treatment were individually placed in three trays (L 440 cm × W 320 cm × H 70 cm). The
210 trays were placed in a rectangular tank (inside dimensions, L 117 × W 78 × H 38 cm) that
211 was aerated and supplied with sand-filtered seawater maintained at approximately 13.5 °C
212 (3L/min/tank, 6 cm depth, flow-through system). To eliminate the effects of the tray
213 position, each tray was rotated in the tank every week. The sea urchins were provided an
214 excess amount of the diet every 2–3 d to allow them to feed until satiation. The uneaten
215 food was collected immediately prior to replenishing the supply with fresh food, and then
216 frozen at −18 °C. At the end of the experiment, the BW of all sea urchins in the six
217 treatments was measured, and the growth rates for each basket were calculated as follows:
218 Specific growth rate (SGR) in BW (%/day) = [ln (final BW) – ln (initial BW)] ×
219 100 / days.
220 The daily seawater temperature in the tank was calculated from the data measured every
221 1 h using a data logger (TR-52i, T&D, Nagano, Japan). The average seawater temperature
222 in the tank during the experiment was 13.8 ± 0.6 °C (mean ± standard deviation).
223
224 2.3.2. Experiment SII: small sea urchins fed with 0–10% gluten diets

225 Sea urchins were subjected to five feeding treatments with 0, 2, 4, 7, and 10% gluten
226 diets (SII-0, SII-2, SII-4, SII-7, and SII-10; Tables 1 and 3). The feeding experiment was
227 conducted from October 6 to November 9, 2021. At the start of the experiment, 102 sea
228 urchins (82–147 mg BW) from Group B were weighed and divided into six groups; one
229 was treated as an initial control and dissected at the start of the experiment (SII-C, 12
230 individuals), and the remaining were used for the five treatments (18
231 individuals/treatment) (Table 3). On November 5, the BWs of all sea urchins in the five
232 treatments were measured, and the growth rate for each basket was calculated. The sea
233 urchins were then placed back in the baskets in the tank and provided an excess amount
234 of fresh *Saccharina longissima* kelp, which was collected from an area facing the Kushiro
235 Field Station in October 2021, until November 9, to replace the gut contents of the urchins
236 fed the artificial diet with kelp. The whole bodies of the sea urchins were then oven-dried
237 at 60 °C for 3 d, and the dry weight was measured. The dried samples for each basket
238 were mixed and used for analyzing the protein content (see Supplementary material S1
239 for details). The dried samples of SII-C were divided into three groups based on average
240 body size, and the mixtures by groups were used for further analysis.
241 The other procedures used were the same as those indicated in experiment SI. The
242 average seawater temperature in the rearing tank was 13.6 ± 1.0 °C.

243

244 2.3.3. Experiment MI: medium sea urchins fed with 5–40% gluten diets

245 Sea urchins were subjected to six feeding treatments with 5, 10, 15, 20, 30, and 40%
246 gluten diets (MI-5, MI-10, MI-15, MI-20, MI-30, and MI-40; Tables 1 and 3). The feeding
247 experiment was conducted from October 20 to November 17, 2021. At the start of the
248 experiment, 102 sea urchins (722–1373 mg body wet weight, BW) from Group B were
249 weighed and divided into seven groups; one was used as the initial control (MI-C, 12
250 individuals), and the others were used for the six treatments (15 individuals/treatment)
251 (Table 3). The urchins in each treatment were housed in three polyethylene baskets at a
252 density of five individuals/basket (three baskets/treatment). The sea urchins in MI-C were
253 dissected at the start of the experiment, and the remaining individuals in the six treatments
254 were dissected at the end of the experiment after BW measurements; all gonads were
255 removed from the test, blotted dry on a paper towel, and weighed using an electric balance.
256 The gonad index (GI) of each individual and the weekly increase in GI for each basket
257 were calculated as follows:

258 $$GI = gonad\ wet\ weight\ \times \frac{100}{BW},$$

259 $$Weekly\ increase\ in\ GI = (final\ GI - initial\ GI)\ \times \frac{7}{days}.$$

260 The gonad tissues were oven-dried at 60 °C for 3 d, and the dry weight was measured.

261 The water content and dry weight of the gonads of each individual were measured, and
262 the weekly increase in the gonad dry matter index (weekly increase in GDI) for each
263 basket was calculated according to Takagi et al. (2022) as follows:

264 $$Gonad\ water\ content\ (\%) = (wet\ weight\ of\ gonad\ tissue -$$

265 $$dry\ weight\ of\ gonad\ tissue) \times \frac{100}{wet\ weight\ of\ gonad\ tissue},$$

266 $$Gonad\ dry\ weight = gonad\ wet\ weight \times \frac{dry\ weight\ of\ gonad\ tissue}{wet\ weight\ of\ gonad\ tissue},$$

267 $$Weekly\ increase\ in\ GDI = (\frac{final\ gonad\ dry\ weight}{final\ BW} - \frac{gonad\ dry\ weight\ of\ MI\text{-}C}{BW\ of\ MI\text{-}C}) \times \frac{7}{days}.$$

268 The gonad protein content was determined for six urchins from each experimental
269 treatment (two urchins from each of the three baskets), and six urchins from the MI-C.
270 Individuals with the second and third largest gonad dry weights from each basket and six
271 individuals with the largest gonad dry weight in the MI-C were selected. Details of protein
272 analysis are provided in Supplementary material S1.
273 The other procedures used were the same as those described in experiment SI. The
274 average seawater temperature in the rearing tank was 13.8 ± 0.6 °C.
275
276 2.3.4. Experiment MII: medium sea urchins fed with 0–10% gluten diets
277 Sea urchins were subjected to five feeding treatments with 0, 2, 4, 7, and 10% gluten
278 diets (MII-0, MII-2, MII-4, MII-7, and MII-10; Tables 1 and 3). The feeding experiment

was conducted from November 24 to December 24, 2021. At the start of the experiment, 87 sea urchins (612–1784 mg BW) from Group B were weighed and divided into six groups; one was used as the initial control (MII-C, 12 individuals), and the others were used for the five treatments (15 individuals/treatment) (Table 3).

The other procedures used were the same as those described for experiment MI. The average seawater temperature in the rearing tank was 13.6 ± 0.9 °C.

2.3.5. Experiment LI: large sea urchins fed with 5–40% gluten diets

Sea urchins were subjected to six feeding treatments with 5, 10, 15, 20, 30, and 40% gluten diets (LI-5, LI-10, LI-15, LI-20, LI-30, and LI-40; Tables 1 and 3). The feeding experiment was conducted from November 19 to December 23, 2021. At the start of the experiment, 120 sea urchins (9.0–18.3 g BW) from Group A were subjected to TD measurement with a vernier caliper (0.01 mm accuracy) and BW measurement using a balance. The sea urchins were divided into seven groups; one was used as the initial control (LI-C, 12 individuals), and the remaining were used for the six treatments (18 individuals/treatment) (Table 3). The urchins in each treatment were housed in three polyethylene cages (L 22 cm × W 15 cm (bottom), L 25 cm × W 17 cm (upper), H 14 cm,

297 with 5 mm × 5 mm mesh on the sides higher than 1.8 cm from the bottom) at a density of
298 six individuals/cage (three cages/treatment). The three cages from each treatment were
299 individually placed in three rectangular tanks (inside dimension, L 117 ×W78 × H38 cm).
300 The tanks were aerated and supplied with sand-filtered seawater maintained at
301 approximately 13.5 °C (3 L/min/tank, 15 cm depth, flow-through system). To eliminate
302 the effects of the cage position, each cage was rotated in the tanks every week. At the end
303 of the experiment, the TD and BW of all sea urchins were measured; the SGR in TD was
304 calculated as follows:

305 SGR in TD (%/day) = [ln (final TD) – ln (initial TD)] × 100 / days.

306 The gonad protein content was determined for six urchins from each experimental
307 treatment (two urchins from each of the three cages) and six urchins from the LI-C.
308 Individuals with GI values close to the average for their cage or the average of the LI-C
309 group were selected.

310 Other procedures used were the same as those described in experiment MI. The
311 average rearing water temperature was 13.5 ± 1.0 °C in one of the tanks and 13.4 ± 1.0 °C
312 in the others.

313

314 2.3.6. Experiment LII: large sea urchins fed with 0–10% gluten diets

315 Sea urchins were subjected to five feeding treatments with 0, 2, 4, 7, and 10% gluten
316 diets (LII-0, LII-2, LII-4, LII-7, and LII-10; Tables 1 and 3). The feeding experiment was
317 conducted from January 14 to February 17, 2022. At the start of the experiment, 72 sea
318 urchins (8.8–13.4 g BW) from Group A were measured for TD and BW. The sea urchins
319 were divided into six groups; one for the initial control (LII-C), and the others for the five
320 treatments (12 individuals/treatment) (Table 3). The urchins in each treatment were
321 housed in two polyethylene cages at a density of six individuals/cage (two
322 cages/treatment); each cage was then placed in two rectangular tanks.
323 Other procedures were the same as those described in experiment LI. The average
324 seawater temperature in the tank was 12.7 ± 1.3 °C.
325
326 2.4. Food and protein intake, and food and protein efficiency
327 The frozen uneaten foods were oven-dried at 60 °C for 72 h after the feeding trials
328 were completed. The weight of the food provided to the urchins and the dry weight of the
329 uneaten food was measured for each basket or cage. To determine the change in diet
330 weight without the influence of sea urchin feeding, each diet used for the feeding
331 experiments was immersed in the rearing tanks for 48 h (three replicates each). The
332 samples were then oven-dried at 60 °C for 72 h. The weight change ratio of each diet (R)

was calculated by dividing the dry weight of the diet after immersion by the weight of the diet before immersion, and was used to calculate the food intake for each basket or cage, as follows:

$$Food\ intake = \frac{(WW_{given} \times R - DW_{left})}{number\ of\ individuals\ in\ basket},$$

where WW_{given} is the total weight of diets provided in the basket (or cage), R is the weight change ratio, and DW_{left} is the total dry weight of uneaten diets left in the basket or cage.

To determine the protein content of the diets after they were fed to the urchins, a seawater immersion test was conducted for each diet. Briefly, 5–8 samples of each diet were immersed in seawater for 24 and 72 h. The diets were subjected to protein analysis before and after 24 and 72 h of immersion (Supplementary material S1). The average protein content in the diets after 24 and 72 h of immersion (n = 2) (Table 1) was used to calculate the protein intake, as follows:

$$Protein\ intake = \frac{(average\ protein\ content \times food\ intake)}{100}.$$

Daily food intake, daily protein intake, feed efficiency (FE), protein efficiency (PE), and protein retention for the experiment SII and gonad protein retention for experiments MI, MII, LI, and LII were calculated for each basket or cage, as follows:

$$Daily\ food\ intake\ (\%/day) =$$

$$\frac{food\ intake \times 100}{[days \times (total\ final\ BW + total\ initial\ BW + Bw\ of\ dead\ animal)/(2 \times number\ of\ individuals\ in\ bastket)]},$$

$$Daily\ protein\ intake\ (\%/day) = \frac{protein\ intake \times 100}{[days \times (total\ final\ BW + total\ initial\ BW + BW\ of\ dead\ animal)/(2 \times number\ of\ individuals\ in\ basket)]},$$

$$FE\ (\%) = \frac{(final\ BW + BW\ of\ dead\ animal - initial\ BW)}{number\ of\ individuals\ in\ basket} \times \frac{100}{food\ intake},$$

$$PE\ (\%) = \frac{(final\ BW + BW\ of\ dead\ animal - initial\ BW)}{number\ of\ individuals\ in\ basket} \times \frac{100}{protein\ intake},$$

$$Protein\ retention\ (\%) = \frac{(final\ body\ protein\ content \times final\ body\ dry\ weight - initial\ body\ protein\ content \times intial\ body\ dry\ weight)}{protein\ intake},$$

$$Gonad\ protein\ retention\ (\%) = \frac{(final\ gonad\ protein\ content \times final\ gonad\ dry\ weight - initial\ gonad\ protein\ content \times initial\ gonad\ dry\ weight)}{protein\ intake}.$$

2.5. Histological Observation.

The gonads of all animals in experiments LI and LII were subjected to histological observation at the end of the experiment. A small piece (approximately 0.2 g) of gonads was fixed in Davidson's solution, dehydrated, and embedded in paraffin wax. Sections with a thickness of 10 μm were prepared, dewaxed, stained with hematoxylin and eosin,

and observed under a light microscope (BH-2, Olympus Corporation, Tokyo, Japan). The gonadal maturities of the animals were classified into five stages as described by Fuji (1960) with minor modifications (Unuma, 2002), as follows: recovering (stage 1, before gametogenesis), growing (stage 2, early gametogenesis), premature (stage 3, mid-gametogenesis), mature (stage 4, late gametogenesis), and spent (stage 5, after spawning).

2.7. Statistical analyses

Statistical analyses were performed using R version 4.1.0. The data were tested for the homogeneity of variance using Levene's test. Significant differences in BW, body dry weight, GI, TD, gonad dry weight, and gonad water content among treatments were analyzed using nested one-way analysis of variance (ANOVA). Tukey's multiple comparison test was performed as a post-hoc test. Significant differences in body (SII) and gonad (MI, MII, LI, and LII) protein contents, protein retention, SGR, weekly increase in GI, daily food intake, daily protein intake, FE, and PE among treatments were tested using the Tukey–Kramer method.

To determine the dietary protein requirement for somatic growth and gonad production, the broken-line regression model, which is used to estimate the protein requirement of sea urchins (Takagi et al., 2022), was used to predict the relationship

387 between the dietary protein content and SGR of BW in experiments SII, MII, and LII,
388 and between the dietary protein content and weekly increase in GDI in experiments MI
389 and LI. Additionally, the relationships between the dietary protein contents and SGRs of
390 TD in experiment LII were predicted to estimate the protein requirement for test growth.
391
392 3. Results
393 3.1. Experiment SI
394 No sea urchins died during the experiment. The average BW increased from 121 mg
395 to > 200 mg in all treatments; that of SI-5 was the largest with 214 mg, although
396 significant differences in the final BW and SGR of BW were not detected among
397 treatments (Tables 3, S1 and S2; Fig. 1B). Thus, the protein requirement for somatic
398 growth of small sea urchins was suggested to be <10%. Daily food intake of SI-5 was
399 significantly higher than that of other treatments ($P < 0.001$; Fig. 2B). Daily protein intake
400 and FE increased with dietary protein increase, and FE of SI-40 was significantly higher
401 than that of SI-5, -10, and -20 ($P < 0.05$; Figs. 2D and S2B). No significant differences
402 were observed in PE among treatments (Fig. S2D).
403
404 3.2. Experiment SII

405 No sea urchins died during the experiment. The average BW increased from 119 mg
406 to 177 mg in SII-0 and to >200 mg in the other treatments. The final BW, body dry weight,
407 SGR of BW, and body protein content of SII-0 were significantly lower than those of the
408 other treatments ($P < 0.05$; Tables 3, S1, S2, and S3; Fig. 1A). FE and PE increased and
409 decreased, respectively, with an increase in dietary protein (Figs. S2A and S2C). The daily
410 food intake of SII-2 and daily protein intake of SII-7 were significantly higher than those
411 of SII-10 and SII-0, respectively ($P < 0.001$; Figs 2A and 2C). Protein retentions of SII-
412 0, -2, and -4 were significantly higher than those of the other treatments ($P < 0.01$; Table
413 S3).
414
415 3.3. Experiment MI
416 No sea urchins died during the experiment. The average BW increased from 1039
417 mg to 1561 mg in MI-5 and to 1462–1496 mg in the other treatments, although significant
418 differences in the final BW and SGR of BW among treatments were not detected (Tables
419 3, S1 and S2; Fig. 1D). Thus, the protein requirement for somatic growth of medium-
420 sized urchins was suggested to be <10%. The GI of all treatments increased from 2.6 to
421 >7.8 at the end of the experiment, and the GI and weekly increase in GI of MI-15 and -
422 20 were significantly higher than those of MI-5 ($P < 0.05$; Table 4; Fig. 3B). The SGR of

423 BW, daily food intake, and PE followed a decreasing trend (Table S2; Figs. 1D, 2F, and
424 S2H); daily protein intake and FE increased with an increase in dietary protein (Figs. 2H
425 and S2F). There were no significant differences in SGRs of BW, gonad dry weight, gonad
426 protein content, and protein retention for gonads among treatments (Tables S1, S2 and
427 S4; Fig. 1D). A significant difference in gonad water content was detected using a nested
428 ANOVA, but not by the Tukey–Kramer method (Tables 4 and S1).
429
430 3.4. Experiment MII
431 No sea urchins died during the experiment. The average BW increased from 1050
432 mg to 1501 mg in MII-0 and to >1700 mg in the other treatments, but there were no
433 significant differences among treatments (Tables 3 and S1). The SGR of BW of MII-0
434 was significantly lower than that of the other treatments ($P < 0.05$; Table S2; Fig. 1C). GI
435 increased in all treatments, and higher dietary protein resulted in higher GI at the end of
436 the experiment (Table 4). Weekly increase in GI, gonad dry weight, gonad water content,
437 and FE were also higher with higher dietary protein (Tables 4 and S4; Figs. 3A and S2E).
438 Thus, the protein requirement for gonad production of medium-sized urchins was
439 suggested to be >10%. No significant differences were observed in protein retention for
440 gonads among treatments (Table S4). The daily food intake and PE decreased with an

increase in dietary protein (Figs. 2E and S2G). The daily protein intake of MII-7 and gonad protein content of MII-2, -4, and -7 were significantly higher than those of MII-0 ($P < 0.05$; Table S4; Fig. 2G).

3.5. Experiment LI

One individual in the LI-40 treatment group died on November 29. The average BW increased from 12.4 g to >14.9 g in all treatments at the end of the experiment. There were no significant differences in the final BW and TD, gonad dry weight, gonad water content, gonad protein content, and specific growth rates in BW and TD among treatments (Tables 3, 5, S1 and S4; Figs. 1F and 1H). GI and weekly increase in GI of LI-20 were significantly higher than those of LI-0 ($P < 0.05$; Table 5; Fig. 3D). The daily food intake decreased, and FE increased with an increase in dietary protein (Figs. 2J and S2J). Protein retention for gonads and PE of LI-15 were significantly higher than those of LI-30 and LI-40 ($P < 0.05$; Table S4; Fig. S2L), and the daily protein intake of LI-15 was significantly lower than that of LI-20, -30, and -40 ($P < 0.05$; Fig 2L). At the end of the experiment, all individuals had ovaries/testes at stages 2 or 3 (Figs. S3A and S3B). The nutritive phagocytes remained predominant in the gonadal acini of all individuals.

459 3.6. Experiment LII

460 One individual in the LI-10 treatment group died on January 31. The BW increased
461 from 11.3 g to 13.6 g in LII-0 and to >14.7 g in the other treatments, with no significant
462 differences among treatments (Tables 3 and S1). Specific growth rates in BW and TD
463 increased with an increase in dietary gluten content from 0 to 4%, then decreased with an
464 increase in dietary gluten content to 10% (Table S2; Figs. 1E and 1G), suggesting the
465 protein requirement for somatic growth of large size urchins to be <10%. Gonad dry
466 weight and GI of LII-0 were significantly lower than those of the other treatments ($P <$
467 0.001, Tables 5 and S4); a similar tendency was observed with the weekly increase in GI
468 (Fig. 3C). Considering the results of experiment LI, the indicated protein requirement for
469 gonad production of large-sized urchins was >5%. The protein retention for gonads and
470 PE decreased and FE increased with an increase in dietary protein (Table S4; Figs. S2I
471 and S2K). The daily food intake of LII-2 and the daily protein intake of LII-7 were higher
472 than those of the other treatments (Figs. 2I, and 2K). At the end of the experiment, all
473 individuals had ovaries/testes at stage 2 or 3 (Figs. S3C and S3D). Nutritive phagocytes
474 remained predominant in the gonadal acini of all individuals.

475

476 3.7. Protein requirement

According to the results of each experiment, data from experiments SII, MII, and LII were used to estimate the protein requirement for somatic growth; those of the experiments MI and LI were used to estimate the protein requirement for gonad production. Broken-line regression analyses between dietary protein content and SGR in BW for small, medium, and large-sized sea urchins predicted the breakpoints of dietary protein to be 2.0% (r^2 = 0.680), 2.2% (r^2 = 0.657), and 2.0% (r^2 = 0.846), respectively (Figs. 4A, 4B and 4C). Analyses of the SGR in TD of large urchins indicated the breakpoints of dietary protein to be 2.2% (r^2 = 0.794) (Fig. 4D). Thus, the protein requirement for somatic growth was approximately 2% regardless of the body size (life stage). Broken-line regression analyses between dietary protein content and weekly increase in the GDI for medium- and large-sized sea urchins predicted the breakpoint of dietary protein to be 12.5% (r^2 = 0.2475) and 15.3% (r^2 = 0.527), respectively, although a significant correlation for the medium-sized urchins was not detected (Figs. 5A and 5B). Thus, the protein requirement for gonad production was ≤15% regardless of the body size (life stage).

4. Discussion

Somatic and gonad growths of *S. intermedius* at different life stages (i.e., before/after

495 gonad differentiation and with/without maturation potential) were investigated in the
496 feeding trials using diets containing 0.6–36.4% protein, with consideration of protein
497 leaching. The broken-line regression analyses on SGRs of BW and TD revealed that the
498 protein requirement for somatic growth was approximately 2%, regardless of sea urchin
499 life stages (Fig. 4). The estimated value of 2% was below the range of protein content in
500 diets (9–50%) used in previous studies that investigated the protein requirement of sea
501 urchins. Analyzing the weekly increase in GDI revealed that the protein requirement for
502 gonad production of medium-sized urchins with semi-transparent gonads (Fuji, 1960)
503 was approximately 13%, without significance, whereas that of large-sized urchins close
504 to the biological minimum size (Fuji, 1960) was approximately 15% (Fig. 5). Thus, the
505 general protein requirement for gonad production was ≤ 15% in both life stages.
506 The protein requirement for gonad production has been investigated in some edible
507 sea urchin species. A comparison of five different diets containing 10–50% protein
508 indicated the protein requirement for gonad production in juvenile *Pseudocentrotus*
509 *depressus* (approximately 1.6 g BW) to be 30% (Akiyama et al., 2001). A previous study
510 on young *Paracentrotus lividus* (approximately 17 g BW) showed that a diet containing
511 30% protein produced the greatest GI increase among six experimental diets with 20–
512 45% protein (Lourenço et al., 2020). The protein requirement of juvenile *Lytechinus*

513 *variegatus* (approximately 4 g BW) was suggested to be 30 and 35% when the diet
514 contained 18 and 12% carbohydrates, respectively (Heflin et al., 2016). A feeding
515 experiment on juvenile *S. intermedius* (approximately 1 g BW) using diets containing 12,
516 18, 24, 30, and 36% protein showed that the GI of sea urchins increased with an increase
517 in dietary protein (Zuo et al., 2017). The protein requirement of juvenile (medium-size
518 group) and young (large-size group) *S. intermedius* estimated in the present study (≤15%)
519 was much lower than the values estimated in previous studies, and was close to that of *M.*
520 *nudus* adults (12%, Takagi et al., 2022), which was estimated after eliminating the
521 influence of protein leaching. Takagi et al. (2022) showed that >40% of the protein
522 content in diets can elute out depending on the binder and protein sources. These results
523 indicate that the protein requirement for gonad production is modest in common to *S.*
524 *intermedius* and *M. nudus*, which further indicates that the protein requirement of sea
525 urchins was overestimated in previous studies where protein leaching was not addressed.
526 The drastic difference in the protein requirement for somatic growth determined in
527 the present study (2%) and that estimated in previous studies (≥20%, Akiyama et al.,
528 2001; Hammer et al., 2006b; 2012; Zuo et al., 2017) cannot be explained by protein
529 leaching alone. The protein content in the diets used in the present study was <9%, which,
530 to the best of our knowledge, has not been tested in previous studies (Akiyama et al.,

2001; Hammer et al., 2006b; 2012; Heflin et al., 2016; Zuo et al., 2017; Lourenço et al., 2020). Similar investigations should be conducted with other species including a dietary protein content of <9%. The protein requirement of *S. intermedius* is markedly low compared with that of other herbivorous aquaculture species, such as abalone *Haliotis discus hannai* (23.3–35.6%, Mai et al., 1995) and milk fish *Chanos chanos* (30%, Hussain et al., 2021). However, the protein requirement levels estimated in the present study were close to the protein content in *Laminariales* kelp (approximately 4–16%, e.g., Agatsuma et al., 2002; Schiener et al., 2015), which is preferred by edible sea urchins, including *S. intermedius* and *M. nudus*, and is commonly used as feed for sea urchin aquaculture (Walker et al., 2015). This suggests that herbivorous sea urchins may have evolved to feed on kelp. Moreover, the protein requirement for somatic growth of *S. intermedius* is close to that of the deposit-feeder sea cucumber *Apostichopus japonicus,* which was suggested to be between 6 and 11% (Bai et al., 2016). The low protein requirement might be a common characteristic of echinoderms.

Aging, coupled with an increase in body size in sea urchins, involves a decrease in assimilation efficiency, and an increase in the energy required for maintenance (Fuji, 1967; Fernandez and Boudouresque, 2002). The higher daily intake of food and protein, FE, PE, and SGR of sea urchins in the experiments SI, SII, MI, and MII compared to

549 those in experiments LI and LII (Figs. 1, 2, and S2) would reflect the changes in
550 assimilation efficiency with aging. Nutrients and energy allocated toward somatic growth
551 decrease and those allocated toward gonad production increase with increasing sea urchin
552 size (e.g., Fuji, 1967; Fernandez and Boudouresque, 2002). The protein retention in the
553 gonads of sea urchins was higher in experiments LI and LII than in experiments MI and
554 MII (Table S3), indicating that protein allocation to the gonads increases with aging.
555 However, there were no large variations in the protein requirement among *S. intermedius*
556 life stages.

557 Nutrient balance in foods regulates the food intake in animals (Simpson &
558 Raubenheimer, 2012). An increase in the food intake with decreasing dietary protein has
559 been demonstrated for *P. lividus* (Lourenço et al., 2020), *M. franciscanus* (McBride et al.,
560 1998; 1999), *M. nudus* (Takagi et al., 2022) and *L. variagatus* (Hammer et al., 2004;
561 2006b) as an attempt to compensate for the lack of available protein in the diet (Hammer
562 et al., 2004). As a result, FE decreases with decreasing dietary protein; this tendency was
563 also observed in the present study (Figs. 2 and S2). Meanwhile, the daily food intake of
564 SII-2 and LII-2 was larger than that of SII-0 and LII-0, respectively. Protein is a vital
565 macronutrient that supplies amino acids, which play a key role in the maintenance of
566 physiological functions, such as cell signaling, appetite stimulation, energy utilization,

567 and immunity, in aquatic animals (Wu et al., 2013; 2014). Mozanzadeh et al. (2018)
568 reported that activities of digestive enzymes, including trypsin, lipase, a-amylase, and
569 carboxypeptidase A, in the sobaity seabream *Sparidentex hasta* fed protein-free diets were
570 significantly lower than those in individuals fed diets containing protein. The extremely
571 low protein content in the 0% gluten diet may have affected the digestive systems of *S.*
572 *intermedius* and impaired food consumption (Figs. 2A and 2I).

573 The estimated protein requirements for somatic growth were approximately 10–15
574 percent point lower than that for gonad production (the present study; Akiyama et al.,
575 2001; Zuo et al., 2017). The difference in protein requirements between somatic and
576 gonad growth may reflect the differences in the protein contents of the test (approximately
577 7%) and gonads (between 40–60%) (Fuji, 1967; the present study). Otero-Villanueva et
578 al. (2004) investigated energy partitioning in *Psammechinus miliaris* and showed that the
579 energy, which is mostly supplied from dietary carbohydrates (Watts et al., 2020; Powell
580 et al., 2022), allocated to the test was greater than that allocated to the gonads. In the
581 present study, crystalline cellulose was used to adjust the protein content of each diet
582 because sea urchins seldom digest cellulose (Watts et al., 2020); cellulase genes are
583 insufficiently expressed in sea urchins (Trenzado et al., 2012) and in their gut microbes
584 (Haditomo et al., 2021), and cellulase activity in the gut is limited (Obrietan, 1991). In

addition, *S. intermedius* has lower degradation activity towards crystalline cellulose than towards phosphoric acid-swollen cellulose (Hasegawa et al., 2012). In the present study, digestible carbohydrate was supplied from starch and kelp powder. When the dietary carbohydrate is insufficient to generate energy, dietary protein is catabolized to meet the energy demand (Heflin et al., 2016). Therefore, we increased the starch content to the extent possible to supply sufficient amounts of energy and justified the content among all diets. In this case, food intake was proportional to carbohydrate intake. Therefore, the high somatic growth and food intake in SII-2, MII-2, and LII-2 may imply that a higher carbohydrate content is more suitable for somatic growth than for gonad production. Similar to that of the protein content, a wide range of carbohydrate levels in sea urchin diets should be included in future investigations involving body and gonad growth while accounting for nutrient leaching.

The amino acid balance of the diet is a key factor affecting the dietary values of animal feed because a deficiency of essential amino acid in food can result in a decrease in growth in some fish species (e.g., Walton et al., 1986; Davies et al., 1997). In the case of fish diets, supplementation with lysine, an essential amino acid for fish, is required when the protein source is changed from fish to wheat gluten (e.g., Davies et al., 1997; Yamamoto et al., 2001). As noted by Takagi et al. (2022), we cannot exclude the

603 possibility that insufficient lysine content may have been a limiting factor, although
604 neither the essential amino acids for sea urchin nor the required levels for each amino
605 acid are known. In the present study, we selected wheat gluten as the protein source to
606 exclude the possibility of overestimating the protein requirement owing to protein
607 leaching from diets, as described by Takagi et al. (2022). However, using a protein source
608 with a better amino acid composition than that of wheat gluten as the primary protein
609 source for diets may cause a shift in the optimum protein levels to values lower than those
610 obtained in the present study.

611 Climate change is suggested to be a major factor responsible for the decrease in *S.*
612 *intermedius* landings in Japan. A decrease in juvenile recruitment of wild *S. intermedius*
613 owing to an increase in seawater temperature has also been reported in previous studies
614 (Gouda et al., 2017; Gouda and Agatsuma, 2020). Moreover, the occurrence of an
615 unprecedented large-scale outbreak of harmful algae in the Pacific coastal waters off the
616 south-eastern coast of Hokkaido during September–November 2021 resulted in mass
617 mortality of the *S. intermedius* populations in this region (Kuroda et al., 2021; Hasegawa
618 et al., 2022). Therefore, *S. intermedius* aquaculture would undoubtedly guarantee its
619 production and supply. The growth of *S. intermedius* until it attains market size requires
620 over three years and includes two steps: somatic growth of small individuals and gonad

production of adults (Unuma and Kayaba, 2015). The cultivation period mostly involves somatic growth. The findings of this study suggest that 2% is the optimal dietary protein content for somatic growth, regardless of the life stage of sea urchins; this indicates that the amount of protein in the diets can be reduced considerably. Additionally, a large difference in protein requirements was observed between somatic and gonad growths, suggesting the possibility of designing diets that preferentially promote body or gonad growth. The findings of this study provide useful information that could help improve the production efficiency of sea urchin aquaculture.

Data Availability

Raw data were generated at the Fisheries Resources Institute. The data that support the findings of this research are available from the corresponding authors upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

ST: conceptualization, methodology, software, validation, formal analysis, investigation,

resources, data curation, writing—original draft, and visualization. YS: writing—review and editing, project administration, and funding acquisition. TU: conceptualization, methodology, validation, resources, data curation, writing—review editing, and supervision. The other authors were involved in the investigation, resource collection, and writing—review and editing.

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862

863 Table 1. Ingredient concentration, proximate composition, and weight change ratio after seawater immersion of the artificial diets with
864 various gluten concentrations fed to *Strongylocentrotus intermedius* in each experiment.

Wheat gluten concentration (%dry weight)	Concentration of other ingredients (%dry weight)			Water (outer percentage)	Proximate composition (%dry weight)				Protein content after immersion[c] (%dry weight)	Weight change ratio[d]	Experiment using the diets
	Cellulose	Potato starch	Kelp[a]		Protein	Lipid	Ash	Carbohydrate[b]			
0	40	50	10	60	0.9	< 0.1	5.0	94.2	0.6	0.37	SII, MII, LII
2	38	50	10	60	2.6	< 0.1	5.1	92.3	1.9	0.41	SII, MII, LII
4	36	50	10	60	4.0	< 0.1	5.1	90.9	3.3	0.39	SII, MII, LII
5	35	50	10	60	5.3	0.1	5.0	89.6	4.5	0.40	SI, MI, LI
7	33	50	10	60	7.8	0.1	5.1	87.1	5.6	0.45	SII, MII, LII
10	30	50	10	60	9.9	0.1	5.0	85.0	9.0	0.35	SI, SII, MI, MII, LI, LII
15	25	50	10	60	14.8	0.1	5.1	80.1	13.0	0.33	SI, MI, LI
20	20	50	10	60	19.4	0.1	5.3	75.2	17.6	0.34	SI, MI, LI
30	10	50	10	60	29.2	0.2	5.0	65.5	25.9	0.35	SI, MI, LI
40	0	50	10	60	37.0	0.2	5.4	57.4	36.4	0.32	SI, MI, LI

865 Artificial diets were cut into moist sheets of approximately 5–8 × 5–8 × 1–3 mm dimension for experiments SI and SII, 8–15 × 8–15 × 2–
866 5 mm for experiments MI and MII, and 20–70 × 10–20 × 2–5 mm for experiments LI and LII.
867 [a] *Saccharina japonica* var. *diabolica* powder.
868 [b] Calculated by subtracting the ash, crude protein, and crude lipid contents from the total dry matter.

869 [c] Mean values of protein content in diets after 24 and 72 h immersion.

870 [d] Calculated by dividing the dry weight of the diet after immersion by the wet weight of the diet before immersion.

871 Table 2. Three size groups of *Strongylocentrotus intermedius* at different life stages

872

	S	M	L
Body weight (g)	0.08–0.16	0.6–1.8	9–18
Age (yr)	0+	0+	1+
Life stage	before gonad differentiation	possessing small five semi-transparent gonads visible to the naked eye	reaching the biological minimum size

873 S, small-sized; M, medium-sized; L, large-sized sea urchins.

874

875

876

877 Table 3. Body weights of *Strongylocentrotus intermedius* fed diets with different protein
878 contents in each experiment.

Experiment	Treatment	Body weight	
		Start	End
Experiment SI	SI-5	120.7 ± 0.3	213.7 ± 15.3
(N_{cage} = 3, n = 18)	SI-10	120.9 ± 0.3	204.1 ± 10.6
19 days	SI-15	120.9 ± 0.5	206.8 ± 10.8
	SI-20	120.9 ± 0.3	202.8 ± 12.8
	SI-30	121.0 ± 0.3	206.9 ± 3.0
	SI-40	121.0 ± 0.2	204.1 ± 8.7
Experiment SII	SII-C	118.9 ± 20.7	-
(N_{cage} = 3, n = 18)	SII-0	118.9 ± 0.8	176.6 ± 16.8[b]
30 days	SII-2	118.9 ± 0.5	225.9 ± 4.4[a]
	SII-4	119.0 ± 0.9	230.2 ± 22.1[a]
	SII-7	119.1 ± 0.4	221.2 ± 11.1[a]
	SII-10	118.9 ± 0.8	209.1 ± 8.6[a]
Experiment MI	MI-C	1038.2 ± 192.7	-
(N_{cage} = 3, n = 15)	MI-5	1039.4 ± 10.1	1561.3 ± 61.4
28 days	MI-10	1039.5 ± 8.2	1495.5 ± 30.2
	MI-15	1038.5 ± 8.9	1492.3 ± 20.0
	MI-20	1039.1 ± 11.6	1476.7 ± 105.2
	MI-30	1039.2 ± 12.9	1481.7 ± 69.7
	MI-40	1040.0 ± 11.2	1461.8 ± 46.8
Experiment MII	MII-C	1049.3 ± 441.7	-
(N_{cage} = 3, n = 15)	MII-0	1050.9 ± 68.3	1501.3 ± 89.7
30 days	MII-2	1050.1 ± 67.8	1700.1 ± 138.0
	MII-4	1048.9 ± 70.4	1764.4 ± 74.3
	MII-7	1048.8 ± 70.3	1752.8 ± 67.4
	MII-10	1049.7 ± 110.4	1731.5 ± 117.0
Experiment LI	LI-C	12.4 ± 1.9	-
(N_{cage} = 3, n = 18)	LI-5	12.3 ± 0.1	14.9 ± 0.4
34 days	LI-10	12.4 ± 0.1	15.3 ± 0.3
	LI-15	12.3 ± 0.0	15.3 ± 0.7
	LI-20	12.4 ± 0.1	15.4 ± 0.4
	LI-30	12.4 ± 0.1	15.6 ± 0.6
	LI-40	12.4 ± 0.3	15.2 ± 0.4

Experiment LII	LII-C	11.5 ± 1.6	-
(N_{cage} = 2, n = 12)	LII-0	11.3 ± 0.1	13.6 ± 0.0
34 days	LII-2	11.4 ± 0.1	14.8 ± 0.2
	LII-4	11.3 ± 0.0	15.0 ± 0.3
	LII-7	11.4 ± 0.1	14.7 ± 0.1
	LII-10	11.3 ± 0.1	14.8 ± 0.2

The measurement units are “mg” in Experiment SI, SII, MI, and MII, and “g” in Experiment LI and LII. Treatment names are abbreviated according to the experiment names and gluten content in diets. SII-C, MI-C, MII-C, LI-C, and LII-C indicate the initial sea urchins used as control that were dissected at the beginning of each experiment (n = 12). Superscript letters indicate significant differences among treatments (P <0.05 using the Tukey–Kramer method). Values represent the mean ± SD of three cages, with the exception of initial controls.

888 Table 4. Gonad indices and gonad water content of *Strongylocentrotus intermedius* fed
889 diets with different protein contents in the experiments MI and MII.
890

Experiment	Treatment	Gonad index	Gonad water content (%)
Experiment MI	MI-C	2.6 ± 1.2	68.6 ± 2.8
(N_{cage} = 3, n = 15)	MI-5	7.8 ± 0.5^{b}	71.1 ± 0.9
	MI-10	9.5 ± 1.2^{ab}	72.6 ± 2.9
	MI-15	11.3 ± 0.6^{a}	71.8 ± 0.7
	MI-20	11.3 ± 1.0^{a}	72.6 ± 1.3
	MI-30	9.9 ± 0.5^{ab}	74.1 ± 0.4
	MI-40	10.2 ± 1.0^{ab}	75.2 ± 1.6
Experiment MII	MII-C	2.3 ± 1.3	73.5 ± 7.2
(N_{cage} = 3, n = 15)	MII-0	4.6 ± 0.2^{c}	67.9 ± 0.8^{c}
	MII-2	8.7 ± 1.7^{b}	69.7 ± 1.3^{b}
	MII-4	11.8 ± 0.6^{a}	70.6 ± 0.3^{ab}
	MII-7	12.9 ± 1.3^{a}	71.1 ± 0.2^{ab}
	MII-10	13.4 ± 1.8^{a}	71.8 ± 0.6^{a}

891 The abbreviations for each treatment are defined in Table 3. Superscript letters indicate
892 significant differences among treatments at the end of the experiment ($P < 0.05$ using the
893 Tukey–Kramer method). Values represent the mean ± SD of three cages, with the
894 exception of initial controls.
895

Table 5. Test diameter, gonad indices, and gonad water content of *Strongylocentrotus intermedius* fed diets with different protein contents in the experiments LI and LII

Experiment	Treatment	Test diameter (mm)		Gonad index	Gonad water content (%)
		Start	End		
Experiment LI	LI-C	29.93 ± 1.79	-	9.0 ± 1.7	70.7 ± 2.1
(N_{cage} = 3, n = 18)	LI-5	29.81 ± 0.24	31.53 ± 0.35	16.3 ± 1.0^{b}	73.3 ± 0.7
	LI-10	29.93 ± 0.11	31.76 ± 0.39	18.3 ± 1.2^{ab}	73.4 ± 0.6
	LI-15	29.81 ± 0.11	31.65 ± 0.43	19.3 ± 0.4^{a}	73.4 ± 0.1
	LI-20	29.95 ± 0.17	31.69 ± 0.21	19.7 ± 0.2^{a}	72.9 ± 0.9
	LI-30	30.11 ± 0.28	31.69 ± 0.41	18.3 ± 1.7^{ab}	73.6 ± 0.3
	LI-40	29.78 ± 0.53	31.25 ± 0.46	17.9 ± 1.3^{ab}	74.4 ± 0.2
Experiment LII	LII-C	29.12 ± 1.53	-	8.57 ± 1.81	76.8 ± 2.5
(N_{cage} = 2, n = 12)	LII-0	29.03 ± 0.00	30.48 ± 0.24	9.76 ± 0.14^{b}	72.8 ± 0.0
	LII-2	29.19 ± 0.15	31.25 ± 0.21	15.61 ± 0.07^{a}	73.3 ± 0.7
	LII-4	29.04 ± 0.11	31.19 ± 0.22	17.02 ± 0.38^{a}	74.0 ± 0.2
	LII-7	28.93 ± 0.00	30.79 ± 0.08	17.03 ± 1.35^{a}	74.3 ± 0.2
	LII-10	28.78 ± 0.03	30.47 ± 0.03	16.39 ± 1.07^{a}	74.0 ± 0.5

The abbreviations for each treatment are defined in Table 3. Superscript letters indicate significant differences among treatments at the end of the experiment ($P < 0.05$ using the Tukey–Kramer method). Values represent the mean ± SD of the three cages, with the exception of initial controls.

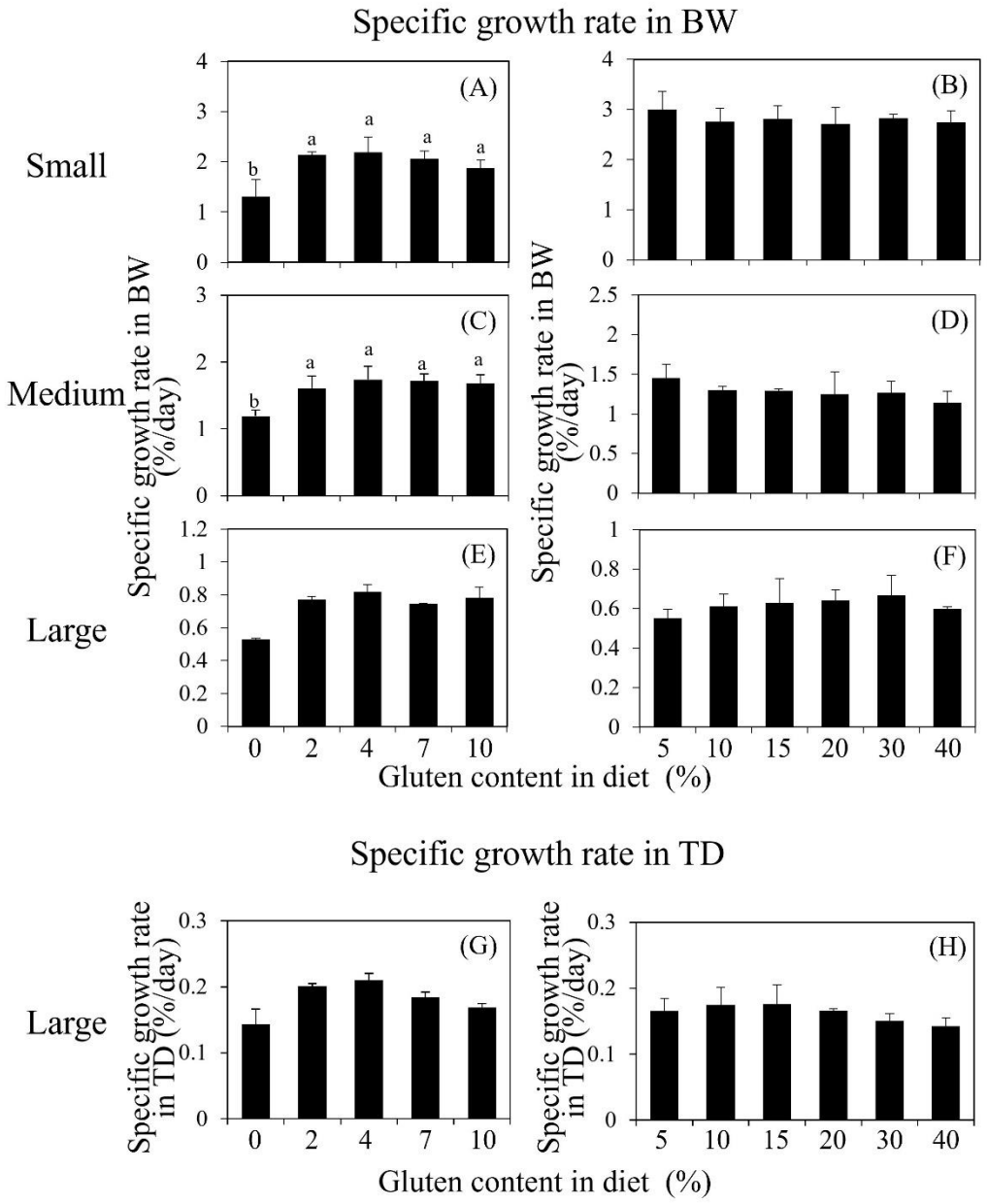

Fig. 1. Specific growth rate in body wet weight (BW) and test diameter (TD) of *Strongylocentrotus intermedius* fed diets with different protein contents. A, C, E, G; sea urchins fed with 0–10% gluten diets. B, D, F, H; sea urchins fed with 5–40% gluten diets. The values are presented as the mean ± standard deviation of the cages, each of which contained six sea urchins for the small- and large-sized groups and five sea urchins for the medium-sized group. Superscript letters indicate significant differences among treatments ($P < 0.05$ using the Tukey–Kramer method).

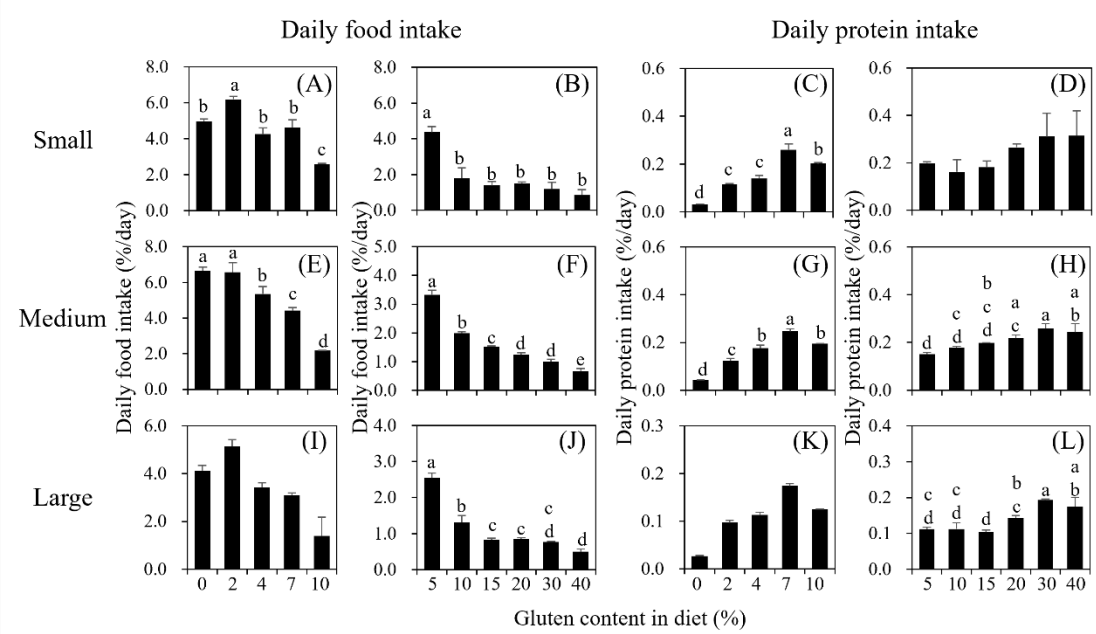

Fig. 2. Daily food (% body wet weight/day) and protein intake (% body wet weight/day) of *Strongylocentrotus intermedius* fed diets containing varying levels of protein. A, C, E, G, I, K; sea urchins fed with 0–10% gluten diets. B, D, F, H, J, L; sea urchins fed with 5–40% gluten diets. The values are presented as the mean ± standard deviation of the cages, each of which contained six sea urchins for the small- and large-sized groups and five sea urchins for the medium-sized group. Superscript letters indicate significant differences among treatments ($P < 0.05$ using the Tukey–Kramer method).

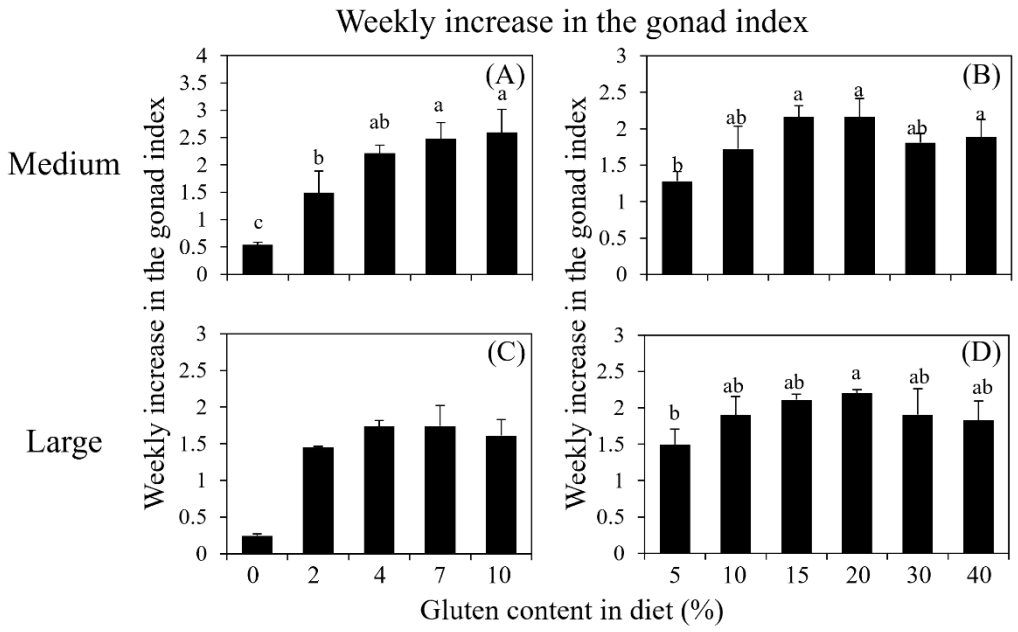

Fig. 3. Weekly increase in the gonad index of *Strongylocentrotus intermedius* fed diets containing varying levels of protein. A, C; sea urchins fed with 0–10% gluten diets. B, D; sea urchins fed with 5–40% gluten diets. The values are presented as the mean ± standard deviation of the cages, each of which contained five sea urchins for the medium-sized group and six sea urchins for the large-sized group. Superscript letters indicate significant differences among treatments ($P < 0.05$ using the Tukey–Kramer method).

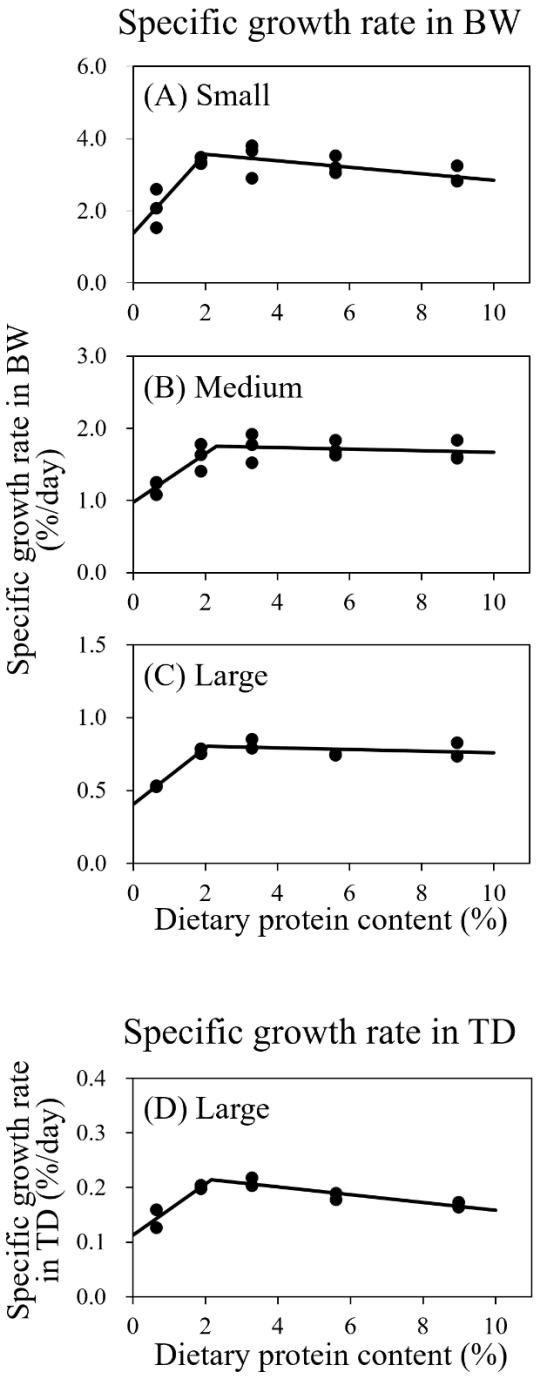

Fig. 4. Relationship between dietary protein content and the specific growth rate in *Strongylocentrotus intermedius* fed with 0–10% gluten diets. A; body weight (BW) of small urchins. B; BW of medium-sized urchins. C; BW of large-sized urchins. D; test diameter (TD) of large urchins. Broken-line regression model (solid line) was used to estimate the protein requirement for somatic growth.

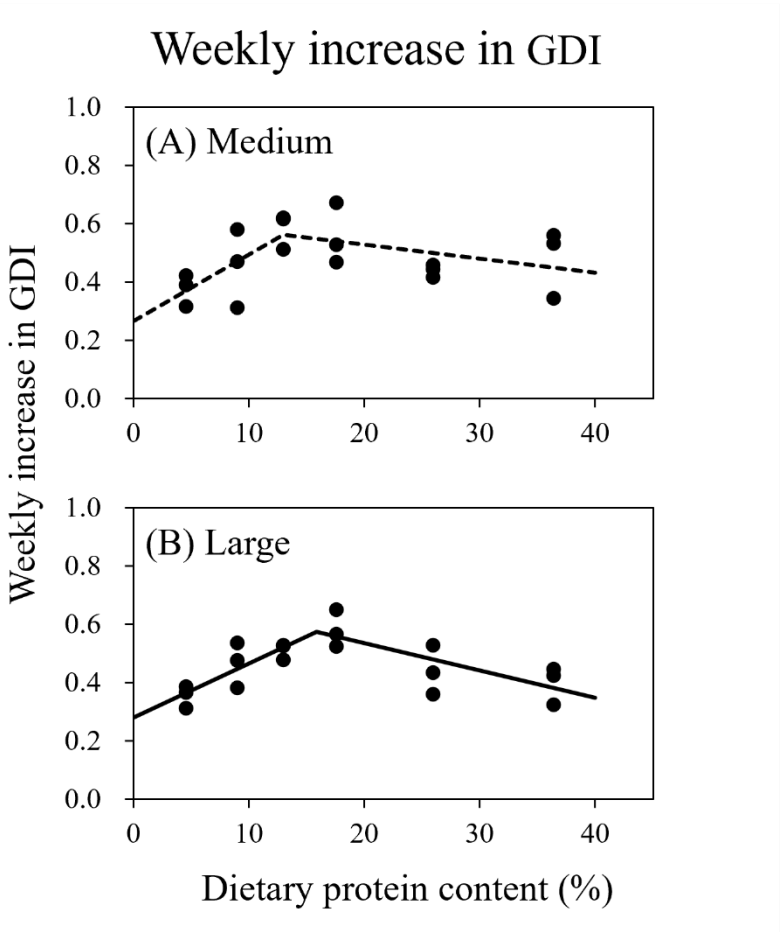

Fig. 5. Relationship between dietary protein content and weekly increase in gonad dry matter index (GDI) of *Strongylocentrotus intermedius* fed with 5–40% gluten diets. A; medium-sized urchins. B; large-sized urchins. Solid and dashed lines indicate the estimated broken-line regression models with and without significance, respectively.

Supplementary materials

Dietary protein requirement for somatic growth and gonad production in the sea urchin *Strongylocentrotus intermedius* at different life stages

Satomi Takagi, Natsuki Hasegawa, Tsuyoshi Watanabe, Yuichi Sakai, Tatsuya Unuma

1. Supplementary Information

Supplementary Information S1. Proximate composition analyses

All samples for proximate analyses were pulverized, and part of the powdered sample was dried at 105 °C 24 h to eliminate all moisture from the samples. The nitrogen contents of the dried diet samples, dried urchin whole bodies from experiment SII, and dried gonads from experiments MI, MII, LI, and LII were analyzed using a CHN analyzer (Flash EA1112, Thermo Finnigan, CA, USA) with acetanilide as a standard based on the combustion method. The crude protein content of the samples was estimated by multiplying the corresponding nitrogen values with 6.25. Crude lipid was extracted by treating the samples with diethyl ether using a Soxhlet apparatus. Ash content was determined by measuring the weight loss in the samples after ignition in a muffle furnace at 550 °C for 12 h. The carbohydrate content was determined by subtracting the ash, crude protein, and crude lipid contents from the total dry matter (AOAC 1990).

Reference

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2. Supplementary Tables

Table S1. Nested ANOVA results of the data from *Strongylocentrotus intermedius* subjected to different treatments under each feeding experiment.

Experiment			*DF*	*SS*	*MS*	*F*	*P*
SI	BW at the beginning	Treatment	5	1	0.2	0.000	1.000
		Treatment: basket	6	2	0.4	0.001	1.000
		Residuals	96	46572	485.1		
	BW at the end	Treatment	5	1390	277.9	0.118	0.988
		Treatment: basket	6	4143	690.5	0.294	0.938
		Residuals	96	225144	2345.2		
SII	BW at the beginning	Treatment	4	0	0.1	0.000	1.000
		Treatment: basket	5	27	5.4	0.016	1.000
		Residuals	80	26228	327.8		
	BW at the end	Treatment	4	33711	3.74	3.740	0.008
		Treatment: basket	5	10609	0.942	0.942	0.459
		Residuals	80	180283			
	BDW	Treatment	4	8548	2137	9.268	< 0.001
		Treatment: basket	5	223	44.6	0.193	0.964
		Residuals	80	18447	230.6		
MI	BW at the beginning	Treatment	5	18	4	0.000	1.000
		Treatment: basket	6	6446	1074	0.028	1.000
		Residuals	78	3038364	38953		
	BW at the end	Treatment	5	131362	26272	0.309	0.906
		Treatment: basket	6	92277	15379	0.181	0.981
		Residuals	78	6621480	84891		
	GI	Treatment	5	128.8	25.767	3.566	0.006
		Treatment: basket	6	20.1	3.357	0.465	0.833
		Residuals	78	563.6	7.226		
	Gonad dry weight	Treatment	5	1291	258.3	0.950	0.454
		Treatment: basket	6	903	150.5	0.553	0.766
		Residuals	78	21213	272		
	Gonad water content	Treatment	5	169.7	33.95	4.290	0.002
		Treatment: basket	6	107.5	17.91	2.264	0.046
		Residuals	78	617.2	7.91		
MII	BW at the beginning	Treatment	4	46	11	0.000	1.000
		Treatment: basket	5	246855	49371	0.206	0.959
		Residuals	65	15568608	239517		
	BW at the end	Treatment	4	703825	175956	0.311	0.870
		Treatment: basket	5	406331	81266	0.144	0.981
		Residuals	65	36793645	566056		
	GI	Treatment	4	807.3	201.83	22.559	< 0.001
		Treatment: basket	5	23.4	4.69	0.524	0.757
		Residuals	65	581.6	8.95		
	Gonad dry weight	Treatment	4	21721	5430	3.567	0.011
		Treatment: basket	5	3080	616	0.405	0.844
		Residuals	65	98968	1523		
	Gonad water content	Treatment	4	135.1	33.78	11.979	< 0.001
		Treatment: basket	5	16.4	3.28	1.163	0.337

		Residuals	65	183.3	2.82		
LI	BW at the beginning	Treatment	5	0.1	0.02	0.004	1.000
		Treatment: cage	6	1.6	0.259	0.055	0.999
		Residuals	96	455.3	4.743		
	TD at the beginning	Treatment	5	1.4	0.286	0.086	0.994
		Treatment: cage	6	4.2	0.703	0.210	0.973
		Residuals	96	320.6	3.34		
	BW at the end	Treatment	5	4.4	0.878	0.137	0.983
		Treatment: cage	6	6.7	1.114	0.174	0.983
		Residuals	95	607.9	6.399		
	TD at the end	Treatment	5	3	0.596	0.177	0.971
		Treatment: cage	6	3.8	0.637	0.189	0.979
		Residuals	95	320.4	3.373		
	GI	Treatment	5	130.1	26.02	4.411	0.001
		Treatment: cage	6	74.5	12.421	2.106	0.060
		Residuals	95	560.3	5.898		
	Gonad dry weight	Treatment	5	0.389	0.078	2.303	0.051
		Treatment: cage	6	0.213	0.036	1.052	0.397
		Residuals	95	3.214	0.034		
	Gonad water content	Treatment	5	23.6	4.727	0.849	0.519
		Treatment: cage	6	15.6	2.594	0.466	0.832
		Residuals	95	529.3	5.571		
LII	BW at the beginning	Treatment	4	0.23	0.057	0.034	0.998
		Treatment: cage	5	0.57	0.114	0.069	0.996
		Residuals	49	81.13	1.656		
	TD at the beginning	Treatment	4	1.9	0.475	0.292	0.882
		Treatment: cage	5	0.39	0.078	0.048	0.999
		Residuals	49	79.82	1.629		
	BW at the end	Treatment	4	14.72	3.679	1.386	0.252
		Treatment: cage	5	1.25	0.25	0.094	0.993
		Residuals	49	130.04	2.654		
	TD at the end	Treatment	4	6.49	1.623	0.904	0.469
		Treatment: cage	5	0.979	0.194	0.108	0.990
		Residuals	49	87.9	1.794		
	GI	Treatment	4	450.9	112.72	28.092	< 0.001
		Treatment: cage	5	18.1	3.62	0.903	0.487
		Residuals	49	196.6	4.01		
	Gonad dry weight	Treatment	4	0.769	0.192	13.358	< 0.001
		Treatment: cage	5	0.038	0.008	0.522	0.759
		Residuals	49	0.706	0.014		
	Gonad water content	Treatment	4	16.76	4.189	2.177	0.085
		Treatment: cage	5	4.94	0.988	0.513	0.765
		Residuals	49	94.3	1.924		

BW, body wet weight; BDW, body dry weight; GI, gonad index; TD, test diameter

Table S2. Specific growth rate in body wet weight of *Strongylocentrotus intermedius* fed diets with different protein contents.

		Specific growth rate in body weight (%/day)
Experiment SI	SI-5	3.0 ± 0.4
(N_{cage} = 3, n = 18)	SI-10	2.8 ± 0.3
19 days	SI-15	2.8 ± 0.3
	SI-20	2.7 ± 0.3
	SI-30	2.8 ± 0.1
	SI-40	2.7 ± 0.2
Experiment SII	SII-0	1.3 ± 0.3[b]
(N_{cage} = 3, n = 18)	SII-2	2.1 ± 0.1[a]
30 days	SII-4	2.2 ± 0.3[a]
	SII-7	2.1 ± 0.2[a]
	SII-10	1.9 ± 0.2[a]
Experiment MI	MI-5	1.5 ± 0.2
(N_{cage} = 3, n = 15)	MI-10	1.3 ± 0.0
28 days	MI-15	1.3 ± 0.0
	MI-20	1.2 ± 0.3
	MI-30	1.3 ± 0.1
	MI-40	1.1 ± 0.1
Experiment MII	MII-0	1.2 ± 0.1[b]
(N_{cage} = 3, n = 15)	MII-2	1.6 ± 0.2[a]
30 days	MII-4	1.7 ± 0.2[a]
	MII-7	1.7 ± 0.1[a]
	MII-10	1.7 ± 0.1[a]
Experiment LI	LI-5	0.55 ± 0.05
(N_{cage} = 3, n = 18)	LI-10	0.61 ± 0.06
34 days	LI-15	0.63 ± 0.12
	LI-20	0.64 ± 0.05
	LI-30	0.67 ± 0.10
	LI-40	0.60 ± 0.01
Experiment LII	LII-0	0.53 ± 0.01
(N_{cage} = 2, n = 12)	LII-2	0.77 ± 0.02
34 days	LII-4	0.82 ± 0.04
	LII-7	0.75 ± 0.00
	LII-10	0.78 ± 0.07

The abbreviations for each treatment are defined in Table 3. Superscript letters indicate significant differences among treatments at the end of the experiment. Values represent the mean ± SD of three cages.

Table S3. Body dry weight, protein content, and protein retention of *Strongylocentrotus intermedius* fed diets containing varying levels of protein in experiment SII.

Experiment	Treatment	Body dry weight (mg)	Body protein content (%)	Protein retention (%)
Experiment SII	SII-C	40.6 ± 6.8	11.0 ± 0.8	–
(N_{cage} = 3, n = 18)	SII-0	56.3 ± 2.8^{b}	9.6 ± 0.2^{b}	68.8 ± 11.03^{a}
	SII-2	78.3 ± 3.0^{a}	10.7 ± 0.5^{a}	65.9 ± 3.33^{a}
	SII-4	81.8 ± 4.1^{a}	11.4 ± 0.3^{a}	67.0 ± 1.30^{a}
	SII-7	82.3 ± 0.8^{a}	11.2 ± 0.2^{a}	36.3 ± 4.18^{b}
	SII-10	79.1 ± 0.7^{a}	11.0 ± 0.3^{a}	42.9 ± 3.07^{b}

The abbreviations for each treatment are defined in Table 3. Superscript letters indicate significant differences among treatments at the end of the experiment. Values represent the mean ± SD of three baskets, with the exception of SII-C (n = 12).

Table S4. Gonad dry weight, gonad protein content, and protein retention for gonad of *Strongylocentrotus intermedius* fed diets containing varying levels of protein in the experiments MI, MII, LI, and LII.

Experiment	Treatment	Gonad dry weight (mg)	Gonad protein content (%)	Protein retention for gonad (%)
Experiment MI	MI-C	9.1 ± 5.1	29.8 ± 2.5	–
(N_{cage} = 3, n = 15)	MI-5	37.1 ± 2.1	33.7 ± 1.2	19.1 ± 2.5
	MI-10	40.3 ± 8.6	35.6 ± 2.2	22.3 ± 5.5
	MI-15	47.9 ± 3.8	33.3 ± 0.6	22.1 ± 4.7
	MI-20	45.5 ± 2.8	32.3 ± 1.1	16.5 ± 3.2
	MI-30	39.0 ± 2.9	35.3 ± 1.3	14.5 ± 0.9
	MI-40	39.9 ± 6.5	34.6 ± 2.0	16.7 ± 4.4
Experiment MII	MII-C	7.0 ± 5.9	34.4 ± 4.0	–
(N_{cage} = 3, n = 15)	MII-0	25.5 ± 4.6^{b}	28.0 ± 1.3^{b}	35.4 ± 17.6
	MII-2	50.4 ± 13.9^{ab}	34.4 ± 0.6^{a}	34.6 ± 13.6
	MII-4	64.8 ± 3.1^{a}	35.5 ± 2.6^{a}	28.3 ± 13.6
	MII-7	70.6 ± 8.4^{a}	32.7 ± 1.1^{a}	21.4 ± 5.8
	MII-10	69.9 ± 14.2^{a}	32.0 ± 1.1^{ab}	26.0 ± 8.5
Experiment LI	LI-C	0.33 ± 0.07	45.8 ± 4.2	–
(N_{cage} = 3, n = 18)	LI-5	0.65 ± 0.04	44.8 ± 2.2	25.2 ± 3.0^{bc}
	LI-10	0.75 ± 0.05	45.8 ± 2.1	34.1 ± 5.4^{ab}
	LI-15	0.78 ± 0.02	45.3 ± 0.6	39.5 ± 3.0^{a}
	LI-20	0.84 ± 0.07	44.0 ± 0.9	27.6 ± 3.3^{bc}
	LI-30	0.74 ± 0.08	47.8 ± 3.5	20.5 ± 3.3^{c}
	LI-30	0.7 0± 0.06	46.4 ± 0.6	18.1 ± 2.7^{c}
Experiment LII	LII-C	0.06 ± 0.03	57.5 ± 6.3	–
(N_{cage} = 2, n = 12)	LII-0	0.36 ± 0.00^{b}	46.5 ± 2.6	110.3 ± 3.0
	LII-2	0.62 ± 0.01^{a}	47.3 ± 1.7	55.0 ± 2.1
	LII-4	0.67 ± 0.03^{a}	49.5 ± 1.4	56.6 ± 2.1
	LII-7	0.64 ± 0.04^{a}	49.8 ± 3.8	56.6 ± 2.5
	LII-10	0.63 ± 0.07^{a}	48.8 ± 2.3	33.9 ± 2.5

The abbreviations for each treatment are defined in Table 3. Superscript letters indicate significant differences among treatments at the end of the experiment. Values represent the mean ± SD of three cages, with the exception of initial controls (n = 12).

3. Supplementary figures

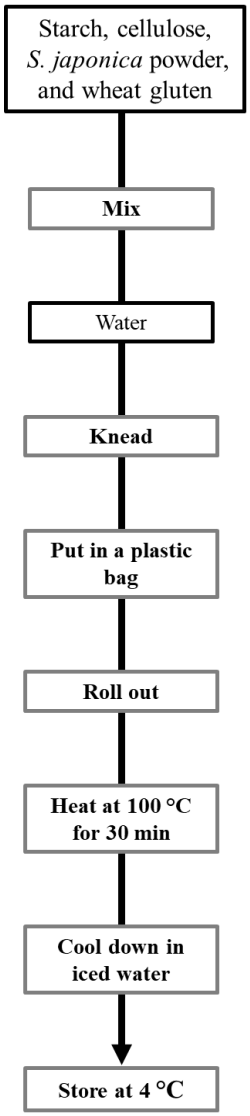

Figure S1. Protocol of artificial diet preparation for *Strongylocentrotus intermedius*. All dry ingredients were thoroughly mixed in a bowl, water was added, and the mixture was kneaded. The mixture was then placed in a plastic bag and rolled out. The starch diet mixture was heated in boiling water for 30 min and cooled in iced water. The prepared diets were stored at 4 °C until further use.

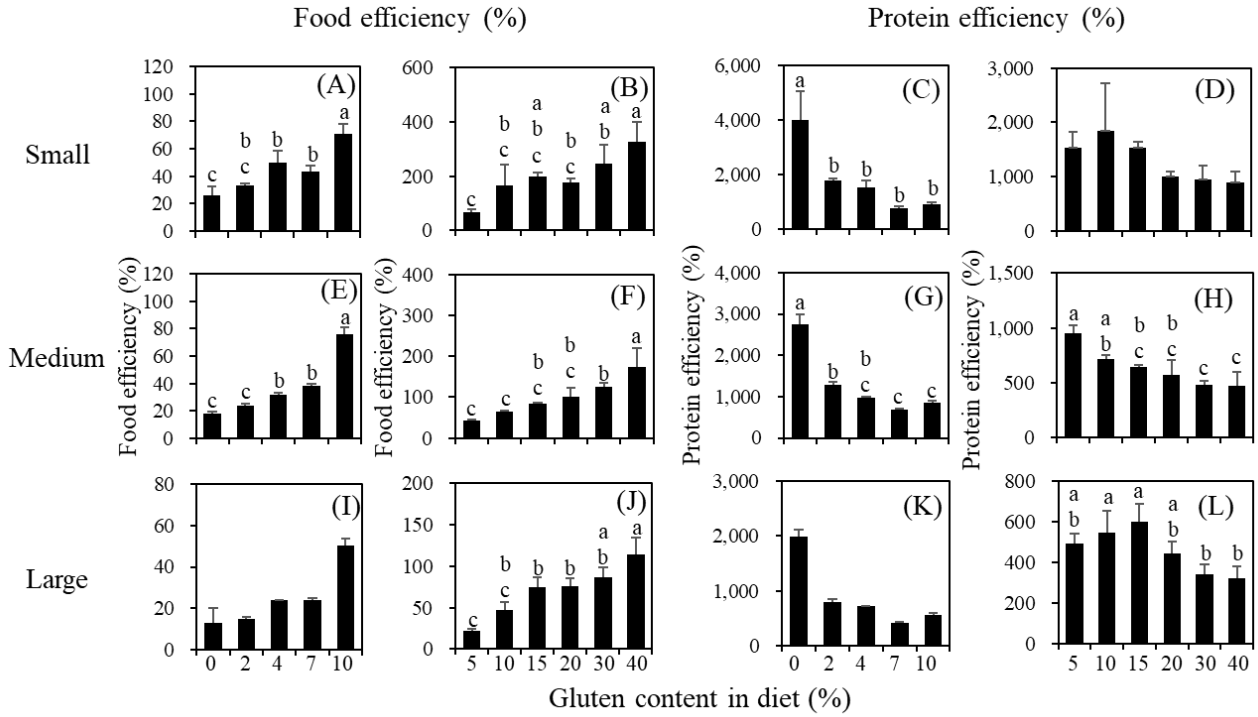

Figure S2. Food efficiency (%) and protein efficiency (%) of *Strongylocentrotus intermedius* fed diets containing varying levels of protein. A, C, E, G, I, K; sea urchins fed with 0%–10% gluten diets. B, D, F, H, J, L; sea urchins fed with 5–40% gluten diets. The values are presented as the mean ± standard deviation of two or three cages, each of which contained six sea urchins for the small- and large- sized groups and five sea urchins for medium-sized group. Superscript letters indicate significant differences among treatments ($P < 0.05$ using the Tukey–Kramer method).

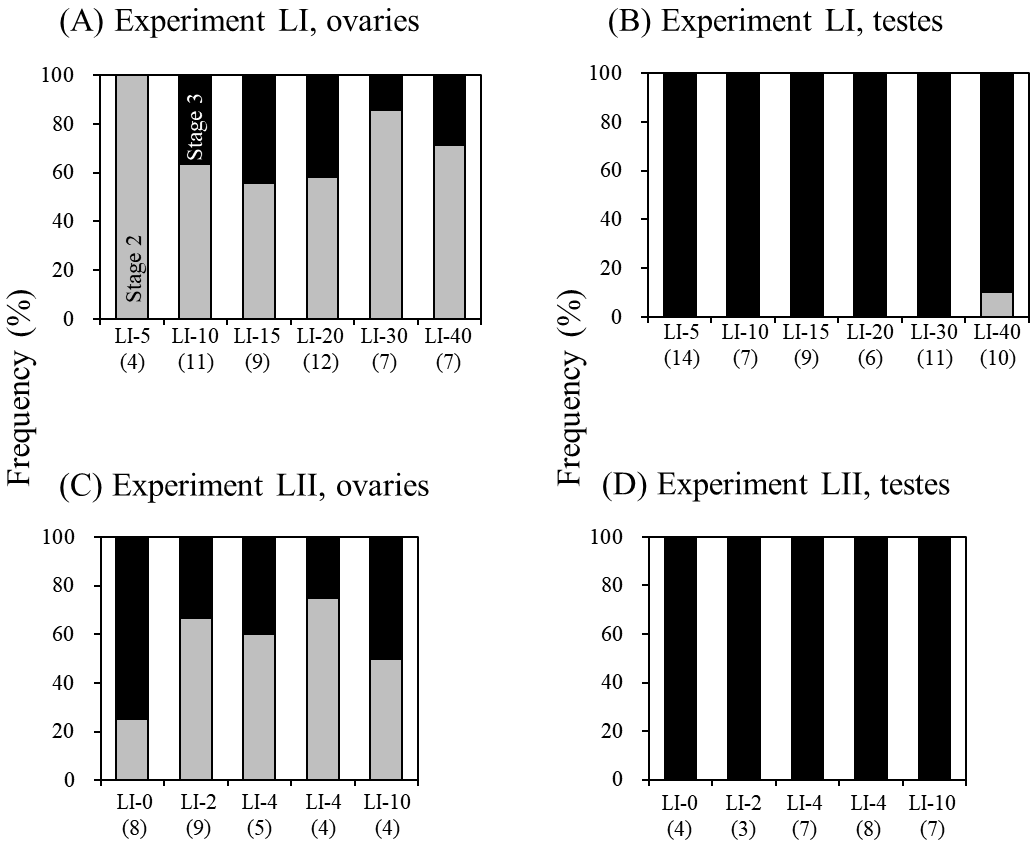

Figure S3. Frequency distribution of the gonad maturation stages of *Strongylocentrotus intermedius* at the end of the experiments LI and LII. A; females in experiment LI. B; males in experiment LI. C; females in experiment LII. D; males in experiment LII. Numerals in parentheses represent the number of individuals.