Metabolic rate and rates of protein turnover in food-deprived cuttlefish, *Sepia officinalis* (Linnaeus 1758)

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Lamarre SG, MacCormack TJ, Sykes AV, Hall JR, Speers-Roesch B, Callaghan NI, Driedzic WR. Metabolic rate and rates of protein turnover in food-deprived cuttlefish, *Sepia officinalis* (Linnaeus 1758). *Am J Physiol Regul Integr Comp Physiol* 310: R1160–R1168, 2016. First published April 6, 2016; doi:10.1152/ajpregu.00459.2015.—To determine the metabolic response to food deprivation, cuttlefish (*Sepia officinalis*) juveniles were either fed, fasted (3 to 5 days food deprivation), or starved (12 days food deprivation). Fasting resulted in a decrease in triglyceride levels in the digestive gland, and after 12 days, these lipid reserves were essentially depleted. Oxygen consumption was decreased to 53% and NH4 excretion to 36% of the fed group following 3–5 days of food deprivation. Oxygen consumption remained low in the starved group, but NH4 excretion returned to the level recorded for fed animals during starvation. The fractional rate of protein synthesis of fasting animals decreased to 25% in both mantle and gill compared with fed animals and remained low in the mantle with the onset of starvation. In gill, however, protein synthesis rate increased to a level that was 45% of the fed group during starvation. In mantle, starvation led to an increase in cathepsin A-, B-, H-, and L-like enzyme activity and a 2.3-fold increase in polyubiquitin mRNA that suggested an increase in ubiquitin-proteasome activity. In gill, there was a transient increase in the polyubiquitin transcript levels in the transition from fed through fasted to the starved state and cathepsin A-, B-, H-, and L-like activity was lower in starved compared with fed animals. The response in gill appears more complex, as they better maintain rates of protein synthesis and show no evidence of enhanced protein breakdown through recognized catabolic processes.

NH4 production; ammonia quotient; cathepsin; triglyceride; digestive gland; proteasome; polyubiquitin

IN THE MARINE ENVIRONMENT, cephalopods are positioned at the top of the invertebrates in terms of size, intelligence, and trophic rank. These molluscs present some functional congruence with fishes, while diverging in important ways, such as whole animal locomotion and metabolic fuel preferences (28). Cephalopods have a “live fast and die young” life strategy. They are mostly carnivorous animals that have a protein-based metabolism (21, 27, 28, 33). Contrary to many other animals, cephalopods do not store substantial amounts of lipids and carbohydrates as energy reserves, although triglycerides in the digestive gland may serve as a short-term fuel to support aerobic metabolism during food limitation. In these animals, surplus energy is instead used for somatic growth, and the protein in the mantle may, thus, be viewed as a form of stored energy (6, 21, 27). A consequence of this metabolic strategy is that, during starvation, cephalopods need to rely rapidly on this protein reserve to sustain metabolism. The physiological response to food deprivation is relatively similar in most vertebrates and invertebrates and is typically described as a three-phase process (4). Phase I is a transient phase occurring during the first few days of food deprivation, where diet-derived carbohydrates, lipids, and proteins are used to maintain basal metabolism. During phase II, the animals mainly mobilize their lipid reserves, with the duration mainly dependent on initial lipid mass. Once lipids are almost depleted, the animals are forced into phase III and oxidize their proteins as a fuel of last resort, and only then, are they considered to be in a true phase of starvation (23). Cuttlefish (*Sepia officinalis*) reach phase III following 7 days of food deprivation (19).

We have previously shown that in the mantle of food-deprived cuttlefish, at a time when digestive gland triglycerides were being mobilized, total RNA levels were decreased and cellular signaling pathways stimulating protein synthesis were disengaged (21). More specifically, the phosphorylation of AKT and 4EBP1 decreased. Phosphorylation of AKT can result in phosphorylation of 4EBP1, leading to an increase of protein synthesis, and dephosphorylation of these proteins should act in the opposite direction (10, 17). Under conditions where triglyceride levels in the digestive gland were almost totally exhausted, some protein degradation pathways in the mantle were activated. Cathepsin A-, B-, H-, and L-like proteases were elevated, as were indices of the ubiquitin-proteasome pathway. Most notably, there were increases in proteasome (β-subunit) and polyubiquitin transcript levels, and polyubiquinated protein (21). These biochemical indices imply that protein synthesis is curtailed during the early stages of food deprivation in the mantle of *S. officinalis*, and as starvation ensues, mantle protein is catabolized. Protein turnover in gill appears to be more complex. The phosphorylation of AKT and 4EBP1 increased under some conditions, suggesting an increase in the rate of protein synthesis. As well, for any given triglyceride level in digestive gland, the total RNA content was higher in food-deprived than fed animals, again consistent with increases in rates of protein synthesis. At the same time, it appears that protein catabolism is activated, as evidenced by increases in proteasome enzyme activity and polyubiquitin transcript levels. Although provocative, the interpretive power...
of the aforementioned experiment (21) is limited because the
initial nutritional state of the animals was not known with
certainty, as these were wild-caught specimens sampled over
two different years, and there was no direct measure of rates of
protein synthesis.

The current experiment aims to further characterize the
effects of starvation on protein metabolism in S. officinalis by
combining whole animal respirometry and rates of ammonia
excretion, measurements of the fractional rate of protein syn-
thesis, activities of enzymes, and expression of genes involved
in protein degradation. This work was conducted with animals
grown since hatching under aquaculture conditions, so the
initial nutritional status was well regulated. Measurements of
oxygen consumption rate (M˙ O2) allow an assessment of the
initial nutritional status was well regulated. Measurements of
protein degradation. This work was conducted with animals
combining whole animal respirometry and rates of ammonia
excretion. The ammonia quotient (A.Q.: M˙ NH4
==
= 0.5°C. Animals that had been fed, fasted for 3–5 days, or starved for 12 days
were transferred into the respirometry chamber at around 1600, and
oxygen consumption was monitored overnight. Oxygen levels were
recorded at 15-min intervals throughout the experiment: 5 min with
the system in closed loop followed by a 10-min flush cycle between
each reading. Animals were housed in the respirometer for 8 to 16 h, and
M˙ O2 returned to baseline levels within the first 2–3 h after
transfer into the system.

Ammonia excretion. NH4 excretion (M˙ NH4) rates for individual
animals were determined by quantifying changes in water NH4 levels
over time in a static tank system. Animals were removed from their
holding tank, weighed, and transferred into darkened 3.0-liter
plastic chambers with continuously aerated seawater at 23.5 ±
0.5°C. A 1.5-ml water sample was collected immediately after
transfer into the chamber and after 60 min. Results from prelimi-
nary studies employing more frequent sampling indicated that
ammonia excretion was pulsatile over short periods, but rates were
consistent when averaged over the full 60-min exposure period.
Ammonia levels were determined by the phenolhypochlorite
method, according to Solórzano (38).

Protein synthesis. The fractional rate of protein synthesis was
measured using the flooding dose method (9) modified to use a stable
isotope tracer (20). Preliminary experiments are required to validate
the methodology, with the key criteria being that 1) the presence of a
high concentration of the amino acid does not affect the rate of protein
synthesis, 2) the labeled amino acid equilibrates rapidly with the
precursor pool, 3) the enrichment of the labeled amino acid remains
unchanged and constant during the incorporation period (9, 20), and 4)
the incorporation of the labeled amino acid in the protein pool is linear
for the duration of the experiment. The method was validated using
fed animals (the animals used for the validation were 33.38 ±
7.81 g, n = 10). Each cuttlefish received an injection of a 150 mM
solution of phenylalanine (PHE) containing 50% ring-D5 L-phenylalanine
(D5-PHE, Cambridge Isotope Laboratories, Tewksbury, MA) in 0.2
μm-filtered seawater at a dosage of 1 ml/100 g body mass. The
labeled amino acid was injected at the base of the arm on the dorsal
side, as previously described for another cephalopod species (5).
Immediately after the injection, the animals were returned to their
respective container. Two animals were sampled after 60, 120, 240,
360, and 480 min following the injection of the tracer. Animals were
killed by performing anesthesia in 5% ethanol in seawater and then
Quickly frozen on dry ice before further processing. Following the
validation experiment, we used the same technique to measure the
fractional rate of protein synthesis in the fed, fasted, and starved animals
(n = 6 per group) using a tracer incorporation time of ~120 min (see
validation results below). All of the tissue-based measurements [i.e.,
triglycerides (TG), enzyme assays, and quantitative PCR] were
conducted using the same animals. Approximately 75 mg of tissue was
read from http://ajpregu.physiology.org/ by 10.220.36.1 on November 9, 2017
homogenized in 1 ml ice-cold perchloric acid (PCA). For the mantle, we used a mini-BeadBeater (BioSpec 31010X with glass beads of 1 mm) at a maximum speed for two bursts of 1 min separated by 30 s on ice, while the gills and digestive gland samples were homogenized using a Polytron homogenizer. The treatment of the homogenized samples to measure protein-bound and free-pool enrichment of phenylalanine was as described before (20). The analyses were performed on an Agilent 5973 mass spectrometer equipped with a 6890 gas chromatograph. The capillary column was a 30-m Agilent DB-5MS (0.25 mm ID, 0.25-μm film thickness). The carrier gas was helium at 1 ml/min. The initial oven temperature was 70°C; 1 min following the injection, the temperature was increased to 280°C at a rate of 25°C/min, and the final temperature was maintained for 5 min (total run time 14.4 min). The mass spectrometer was operated in SIM mode with m/z 300 and 305 ions selected for PHE and D3-PHE, respectively. Fractional rates of protein synthesis (kr, %/day) were calculated from the phenylalanine enrichment of the protein pool [Sg = [D3-PHE]/([D2-PHE] + [PHE])] and the enrichment of the free-pool [Sf = [D3-PHE]/([D2-PHE] + [PHE])], according to kr = 100·[(Sf/Sg) - (t2 - t1)]/(1440t2 - t1) where Sg is the final protein bound D5-PHE enrichment and Sf is the average incorporation at an earlier time (9, 13, 20).

In vitro protein degradation pathways. Tissue samples were homogenized in 5 volumes of ice-cold 50 mM Tris buffer containing 0.1 mM EDTA and 0.007% β-mercaptoethanol (pH 8.0) using a Polytron homogenizer. The samples were centrifuged 13,000 g at 4°C for 60 min, and then the protein concentration was measured using a Bradford assay kit from Bio-Rad. The chymotrypsin-like activity of the 20S proteasome was measured using a microplate fluorescence assay (21). Briefly, each well contained 100 μl of 100 mM Tris buffer with 0.0285% SDS, 40 μM LLVY-AMC, and 50 μg of supernatant protein. Blanks were prepared by adding MG-132 at a final concentration of 50 μM. The MG-132-sensitive activity is reported in relative fluorescence units min⁻¹ μg protein⁻¹. For the various protease assays, the homogenates were diluted to 1% using ice-cold homogenization buffer. Cathepsin-like proteases activities were measured using the McIlvaine’s buffer system (24), and the activity of cathepsin-like proteases was determined in medium containing 20 mM homogenization buffer. Cathepsin-like proteases activities were measured using the M-MLV reverse transcriptase [200 U (Invitrogen/Life Technologies)] with the manufacturer’s first-strand buffer (1X final concentration) and DT (10 mM final concentration) at 37°C for 50 min.

Quantitative PCR analysis of ubiquitin-proteasome pathway related genes. The sequences of all primer pairs used in qPCR analyses are presented in Table 1. Primer sequences for polyubiquitin and proteasome (β-subunit) were reported previously (21). The additional primer sequences were based on the following sequences from GenBank: ubiquitin-activating enzyme (FO182945), ubiquitin-conjugating enzyme (HM157280), elongation factor 1-α (HM157271), and cleavage and polyadenylation specificity factor (CPSF) (HM157279). Each primer pair was quality tested to ensure that a single product was amplified (disociation curve analysis) and that there was no primer-dimer present in the no-template control. Amplicons were electrophoretically separated on 2% agarose gels and compared with a 1k bplus ladder (Invitrogen/Life Technologies) to verify that the correct size fragment was being amplified. Amplification efficiencies (30) were calculated using cDNA synthesized from gill and mantle from two fed, two short-fasted, and some (16, 21) fed animal samples. Transcript levels were measured for four candidate normalizers [CPSF, eukaryotic translation initiation factor (ETIF), and 16S rRNA] using cDNA representing 10 ng of input total RNA.

Transcript levels of the genes of interest (GOIs) were normalized to two endogenous control genes. To select these endogenous controls, transcript levels were measured for four candidate normalizers [CPSF, EF1-α, eukaryotic translation initiation factor (ETIF), and 16S rRNA] using cDNA representing 5 ng of input total RNA synthesized from gill and mantle from two fed, two short-fasted, and some (16, 21) fed animal samples. Transcript levels were measured for four candidate normalizers [CPSF, EF1-α, eukaryotic translation initiation factor (ETIF), and 16S rRNA] using cDNA representing 5 ng of input total RNA synthesized from gill and mantle from two fed, two short-fasted, and some (16, 21) fed animal samples.

**Table 1. Primers used in qPCR studies**

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Direction</th>
<th>Nucleotide Sequence (5'-3')</th>
<th>Efficiency, %</th>
<th>Amplicon Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubiquitin-activating enzyme (E1)</td>
<td>Forward</td>
<td>cccgtatggagctttgagctttg</td>
<td>97</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>gcaaggcastcagcttgtgatca</td>
<td>97</td>
<td>136</td>
</tr>
<tr>
<td>Ubiquitin-conjugating enzyme (E2A)</td>
<td>Forward</td>
<td>atgaccagttttagctgccacgc</td>
<td>97</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>atgggtgctggctgtttgggt</td>
<td>97</td>
<td>122</td>
</tr>
<tr>
<td>Polyubiquitin</td>
<td>Reverse</td>
<td>cccctctctctctcgagcgggcgcgc</td>
<td>93</td>
<td>70</td>
</tr>
<tr>
<td>Proteasome β</td>
<td>Reverse</td>
<td>tggctggcagtttctctcagctgccagc</td>
<td>96</td>
<td>102</td>
</tr>
<tr>
<td>Elongation factor 1-α</td>
<td>Reverse</td>
<td>gccacagtcctctgcggccaggtc</td>
<td>95</td>
<td>104</td>
</tr>
<tr>
<td>Cleavage and polyadenylation specificity factor</td>
<td>Forward</td>
<td>cagctggcaggggctgttttg</td>
<td>97</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ggctggcaggggctgttttg</td>
<td>97</td>
<td>136</td>
</tr>
</tbody>
</table>

*a Amplification efficiencies were calculated using a five-point 1:3 dilution series starting with cDNA representing 10 ng of input RNA.*
two starved S. officinalis. C₄ values were analyzed using geNorm to select the most stably expressed transcripts. Using this software, EF1-α (geNorm M = 0.34) and CPSF (geNorm M = 0.36) were determined to be the most stable.

Transcript (mRNA) expression levels of the GOIs were then assessed by qPCR. In all cases, cDNA representing 5 ng of input RNA was used as a template in the PCR reactions. On each plate, for every sample, the target gene and endogenous controls were tested in triplicate, and a plate linker sample (i.e., a sample that was run on all plates in a given study) and a no-template control were included. The relative quantity (RQ) of each transcript was determined using the Viia 7 Software Relative Quantification Study Application (version 1.2.3) (Applied Biosystems/Life Technologies), with normalization to CPSF and EF1-α transcript levels, and with amplification efficiencies incorporated. For each GOI, the sample with the lowest normalized expression (mRNA) level was set as the calibrator sample (i.e., assigned an RQ value = 1).

Statistical analysis. All values are expressed as means ± SE and were compared using one-way ANOVA and Tukey post hoc test using GraphPad Prism 6. The data were log₁₀ transformed when necessary. All differences were considered significant when P < 0.05.

RESULTS

Nutritional status. As food deprivation persisted, the digestive gland TG content decreased significantly among the fed, short-term fasted (3–5 days of food deprivation 28% of the fed group), and starved (12 days of food deprivation, 1% of the fed group) S. officinalis (Fig. 1) (ANOVA, F₂₀.₁₉ = 15.64; P < 0.001).

Oxygen uptake and ammonia excretion. Food deprivation was associated with a decrease in MO₂ to 53% of the fed group (Fig. 2, top) (n = 6 per group, F₂₀.₁₄ = 12.56, P < 0.001). The duration of the food restriction period (3, 5, or 12 days) did not have a significant effect on MO₂. Short-term (3–5 days) food deprivation was associated with a decrease in MNH₄ to 36% of the fed group. As the food restriction (12 days) persisted, MNH₄ returned to control levels (Fig. 2, bottom). Because MNH₄ and MO₂ were not measured in the same animals, the A.Q. was determined by calculating MNH₄ using group averages of each measurements. A.Q. in the control group was 0.285, and following three to five days of fasting decreased to 0.171. After 12 days of food deprivation, the A.Q. increased to 0.426.

Validation of protein synthesis. The flooding dose technique can only be used when the four criteria underlying the technique are met. The first validation criterion, that a high concentration of phenylalanine does not affect the rate of protein synthesis, could not be tested in this experiment, but we assumed that it was met as usually observed in vertebrates and invertebrates (8). Injected D₅-PHE rapidly flooded the tissues to reach ~30% enrichment of the free phenylalanine pool in both the mantle and the gills (Fig. 3, A and B), thus fulfilling the second criterion that the tracer must equilibrate rapidly with the precursor pool.

The third criterion of sustained enrichment of the tracer was met since Sₕ remained elevated for a period of over 480 min in the mantle (regression line did not deviate from 0, F₁₈,₈ = 5.35, P > 0.05), while a slight decrease was observed in the gills (slope −0.04 ± 0.01%/min, F₁₈,₈ = 12.66, P = 0.007). The fourth criterion for validation of the technique was also met, as Sₕ increased in a linear fashion in the two tissues (Fig. 3, C and D). We deemed that the three last criteria required for the validation of the flooding dose technique were fulfilled. From these data, in further experiments, we elected to use an incorporation period of 120 min, which provided sufficient time for the tracer to accumulate in the protein pool to a detectable level while minimizing the decrease of Sₕ and recycling of the tracer due to protein degradation.

Protein synthesis in fed, fasted, and starved cuttlefish. The fractional rate of protein synthesis in the mantle and gills of fed, fasted, and starved cuttlefish was measured (Fig. 4). In all groups, the rate of protein synthesis was ~8–9 times higher in the gills compared with the mantle. The effect of fasting was similar in the two tissues; food deprivation decreased the rate of protein synthesis to 25% of that observed in the fed animals. As fasting progressed into starvation, there was no further significant change in the rate of protein synthesis in mantle; however, in gill, the rate of protein synthesis significantly
increased to a level that was 45% of the fed group. We also attempted to measure the rate of protein synthesis in the digestive gland; however, $S_a$ decreased considerably during the 120-min incorporation period (data not shown). After the incorporation period, $S_a$ was only 3.24 ± 1.1% in the digestive gland of the fed animals, while it was still 26.0 ± 5.30% in the starved animals. As such, it was impossible to accurately measure the rate of protein synthesis in digestive glands of the fed animals.

**In vitro protein degradation enzyme activities.** The enzyme activity of two classes of cathepsins (pH 2.5 and pH 5.5), the calpain-like proteases, and the 20S proteasome was measured in the fed, fasted, and starved animals (Table 2). For the cathepsins at pH 2.5, the only observable difference was in the

![Graph](image1)

**Fig. 4.** Fractional rate of protein synthesis ($k_s$) in mantle and gill of *S. officinalis* that were either fed, fasted (3 days of food deprivation), or starved (12 days of food deprivation). Values are given as means ± SE; $n = 6$. Different letters indicate significant difference ($P < 0.05$).

**Table 2.** Proteases enzyme activity in gill, mantle, and digestive gland of *Sepia officinalis* that were either fed or starved for 7 days

<table>
<thead>
<tr>
<th></th>
<th>Fed</th>
<th>Starved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cathepsin 2.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gill</td>
<td>3555 ± 278.0</td>
<td>3887 ± 225.6</td>
</tr>
<tr>
<td>Mantle</td>
<td>7175 ± 290.0</td>
<td>6941 ± 159.5</td>
</tr>
<tr>
<td>Digestive gland</td>
<td>11767 ± 152.8</td>
<td>8644 ± 184.8*</td>
</tr>
<tr>
<td>Cathepsin 5.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gill</td>
<td>16012 ± 257.4</td>
<td>13291 ± 595.6*</td>
</tr>
<tr>
<td>Mantle</td>
<td>11369 ± 326.4</td>
<td>14232 ± 1256*</td>
</tr>
<tr>
<td>Digestive gland</td>
<td>216622 ± 11939</td>
<td>116893 ± 9299*</td>
</tr>
<tr>
<td>Calpain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gill</td>
<td>21213 ± 1167</td>
<td>20545 ± 1126</td>
</tr>
<tr>
<td>Mantle</td>
<td>35159 ± 6049</td>
<td>36797 ± 4227</td>
</tr>
<tr>
<td>Digestive gland</td>
<td>247236 ± 23462</td>
<td>259753 ± 11602</td>
</tr>
<tr>
<td>20S Proteasome</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gill</td>
<td>615847 ± 66536</td>
<td>676633 ± 29758</td>
</tr>
<tr>
<td>Mantle</td>
<td>101810 ± 7318</td>
<td>87803 ± 10310</td>
</tr>
<tr>
<td>Digestive gland</td>
<td>962202 ± 149639</td>
<td>552593 ± 25005*</td>
</tr>
</tbody>
</table>

*Values are expressed as means ± SD ($n = 6$). Data are expressed as fluorescence units·min$^{-1}$·mg tissue$^{-1}$ for cathepsin pH 2.5, cathepsin pH 5.5, and calpain: fluorescence units·min$^{-1}$·50 μg protein$^{-1}$ for 20S proteasome. *Significant difference between fed and starved cuttlefish ($P < 0.05$).
digestive gland where the starved *S. officinalis* had lower enzyme activity. For the cathepsins at pH 5.5, we observed differences in the three studied tissues; however, the direction of these differences varied. Enzyme activity was significantly lower in gills and digestive gland of the starved animals with respect to the fed group but was higher in the mantle of the starved than the fed *S. officinalis*. There was no effect of starvation on the calpain-like proteases. The 20S proteasome activity was unaffected by food deprivation in the gills and mantle but was significantly lower in the digestive gland of the starved than fed animals.

Transcript levels of genes related to the ubiquitin-proteasome pathway. UBE1 transcript levels were not affected by food deprivation (Fig. 5A). UBE2A transcript levels were significantly higher in mantle of the 3 days fasted than either fed or starved animals (Fig. 5B); however, there was no change in gill. Polyubiquitin transcript levels were transiently elevated in the gills of the fasted *S. officinalis*, while this increase was observed in the mantle of the starving animals (Fig. 5C). Finally, proteasome (β-subunit) transcript levels decreased in both the gills and mantle of the starving animals with respect to the fed or fasted groups (Fig. 5D).

**DISCUSSION**

**On-board protein ultimately fuels aerobic metabolism.** Consistent with previous studies on food restriction in cephalopods, the triglyceride stores of the digestive gland were decreased to one-third that of the fed *S. officinalis* after 3 days of fasting and were virtually depleted following 12 days of fasting (6, 21). Following 3–5 days of fasting, at a temperature of 21°C, MO₂ was about one half that of the fed animals and remained the same during starvation. Consistent with this, in a similar experiment, Grigoriou and Richardson (11) found that, at a lower temperature of 15°C, metabolic rate began to differ from that of fed animals after ~10 days of food deprivation. The MNH₄ reported here are in the same range as that of the Chinese cuttlefish (*Sepia maindroni*) (42); however, lower rates of MNH₄ for *S. officinalis* were reported earlier (2). We have no explanation for the discrepancy, but we believe that the values reported here for this population of *S. officinalis* are accurate as MNH₄ are compatible with MO₂. The rate of ammonia excretion returned to the fed level after the TG reserves were exhausted. This is in agreement with the view that, when fasting, cephalopods switch from an amino acid-dominated metabolism to a lipid-dominated metabolism by mobilizing the triglycerides stored in the digestive gland (6, 21, 27). This assertion is further supported by the A.Q. of the fed animals that is close to the theoretical maximum calculated to be 0.27 to 0.33 (18) for aerobic oxidation of amino acids. This suggests that virtually 100% of their energy needs are met using amino acids during resting metabolism. In the fasted *S. officinalis*, the A.Q. reveals that ~50% of their aerobically based metabolism is met using amino acids as a fuel source. The source of the amino acids is probably body protein. Cephalopods have very little carbohydrate stores (28, 39), and on the basis of the triglyceride decrease in the digestive gland in food-deprived cephalopods (this study; Refs. 6, 21, 33), the latter likely contributes to the remaining ~50% of aerobic energy metabolism. Following the depletion of triglycerides in the digestive gland after 12 days of starvation, the A.Q. goes above 0.27, which suggests that aerobic metabolism is met almost exclusively using amino acids, and further that anaerobic degradation of amino acids is ongoing and/or that the carbon backbone of deaminated amino acids is used in anabolic reactions (18). We are not aware of any other study in which A.Q. went above 0.27 in aerobic conditions. This novel finding though must be considered with caution as MO₂ and MNH₄ were determined on different animals, but, nevertheless, it is a
provocative discovery that requires confirmation. Regardless, the changes in triglyceride content in the digestive gland along with the decrease and subsequent increase in \( \text{MNH}_2 \) leads us to conclude that the current experimental groups were suitable to test the effects of the duration of the restriction period on protein metabolism. Furthermore, in the starved group, there must be catabolism of body protein, presumably from mantle, given it is by far the largest tissue by mass.

**Control of protein synthesis.** Prior to our study, the fractional rate of protein synthesis had only been measured in two species of cephalopods; the common octopus (*Octopus vulgaris*) (14) and the Southern dumpling squid (*Euprymna tasmanica*) (5, 25, 26), but never in a cuttlefish. Our work is the first to measure the fractional rate of protein synthesis in *S. officinalis*. The flooding dose technique was validated by using a time course and proved to respect all the assumptions of the technique: injected isotope rapidly flooded the tissues, the free pool of phenylalanine remained elevated for the course of the experiment, and phenylalanine enrichment of the protein pool increased in a linear fashion. The fractional rates of protein synthesis in mantle and gill of fed and food-deprived *S. officinalis* are almost identical to what Houlihan et al. (14) calculated for an octopus growing at 6%/day or having a growth rate of zero, respectively. The rates of protein synthesis in *S. officinalis* are lower than those in the southern dumpling squid (5), the only other decapod cephalopod for which comparable data are available. This finding is likely to be a result of the fact that the squid were much smaller than the animals used here (2.8 g and 9.01 g vs. 48.99 g in this experiment) and that these two groups have very different life histories and growth profiles. It was impossible to measure the rate of protein synthesis in the digestive gland because the specific enrichment of free amino acid pool decreased too quickly to allow calculation of the rate of protein synthesis in the fed animals (data not shown). This suggests that the fed animals were still in a postprandial state 24 h following their last meal. Information on the time needed to digest a meal in cephalopods is scarce; however, it is generally accepted that these animals are geared for rapid digestion (36). Furthermore, the digestive gland may be involved in providing precursors for melanin synthesis for the production of ink by the ink gland. In this pathway, phenylalanine is hydroxylated to tyrosine that is then used for the biosynthesis of melanin by the ink gland (29). Thus, it is likely that a great proportion of the tracer phenylalanine was converted to labeled tyrosine, which was not detected by our mass spectrometry analysis.

The measured decrease in rates of protein synthesis in mantle are consistent with previously measured decreases in starved cuttlefish of the phosphorylation of AKT and 4EBP1, which are components of cellular signaling pathways controlling protein synthesis (21). Similarly, the increase in the rate of protein synthesis in the gill as the triglyceride stores in the digestive gland are depleted matches the observed increase in the phosphorylation of 4EBP1. These observations are important beyond *S. officinalis* per se because they help confirm that deductions made from the biochemical analysis of cellular signaling pathways concerning the rate of protein synthesis are valid (15, 21, 34, 35).

**Indices of protein catabolism.** The findings that digestive gland triglyceride reserves are used to the point of depletion, followed by an increase in \( \text{NH}_4 \) excretion with an exceptionally high A.Q. value provides compelling evidence that during food deprivation, *S. officinalis* mainly relies on amino acids from onboard protein to fuel their metabolism. Therefore, it is of interest to learn how protein catabolism is regulated. It is generally accepted that protein stored in the mantle is the primary source of amino acids during starvation (6, 21, 27, 28). The cathepsin A-, B-, H-, and L-like enzyme activity is stimulated in the mantle during starvation while the activity of the other measured proteases remains unchanged. This pattern was previously noted in starving *S. officinalis* (21) and strengthens the putative role of the mantle as a source of amino acids through the degradation of protein involving cathepsin-like proteases. The ubiquitin-proteasome system is a highly regulated protein degradation pathway that requires the coordinated work of many proteins (12) and might also be involved in the food deprivation response. The enzyme activity of the 20S proteasome was not affected by starvation in mantle, again consistent with previous findings (21), but this information alone is not sufficient to declare that this pathway does not play a role in protein catabolism. Accordingly, we measured the transcript levels of UBE1, UBE2A, polyubiquitin and the proteasome (\( \beta \)-subunit). UBE1 transcript levels were not influenced by food restriction; however, UBE2A transcript levels showed a transient elevation in mantle of fasting animals. This protein is responsible for the ubiquitination of the proteins recognized by E3 proteins for degradation (1, 31). After 12 days of food restriction, polyubiquitin transcript levels increased 2.3-fold, once more consistent with earlier findings that reported a massive increase in polyubiquinated protein (21). Proteasome (\( \beta \)-subunit) transcript levels, however, decreased, which is in contrast to our earlier work (21). Overall, the current data set does not convincingly reveal an enhanced ubiquitin-proteasome system in mantle during food deprivation, although the significant increase noted here and the five-fold increase in polyubiquitin transcript levels previously reported (21) warrant further investigation.

Gill appears to present a more complex situation with respect to protein breakdown just as it does for protein synthesis. Cathepsin A-, B-, H-, and L-like activity and the proteasome (\( \beta \)-subunit) transcript levels were lower in starved animals than the other two groups. This suggests decreased rates of protein breakdown at this time point. The transient increase in polyubiquitin transcript levels in the fasted animal suggests similar or even decreased rates of protein breakdown in gill between starved and fed animals. The very clear and significant increase in polyubiquitin transcript levels upon the transition from feeding to fasting confirms earlier findings (21) and implies a transient elevation in the ubiquitin-proteasome system.

In the digestive gland, the activities of the cathepsins and the 20S proteasome were all lower in starved than in fed animals. The simplest explanation for this is that by the time the animals entered the starvation state, any protein that could be mobilized in the tissue had already been catabolized.

**Perspectives and Significance**

Consistent with numerous studies on cephalopods, aerobic metabolism in fed animals is fueled primarily by dietary metabolism. Furthermore, in the starved group, there must be catabolism of body protein, presumably from mantle, given it is by far the largest tissue by mass.
protein. Upon food deprivation, aerobic metabolism is decreased. \( \text{NH}_4 \) production is decreased to a relatively greater extent than \( \text{MO}_2 \), indicating that lipids are being called upon, thus initially sparing body protein. As food deprivation persists, lipids of the digestive gland are depleted, and body protein serves as the aerobic metabolic fuel. This is evidenced by an increase in \( \text{MNH}_4 \) resulting in an A.Q. that fully supports \( \text{MO}_2 \) and tentatively even leads to an excess production of \( \text{NH}_4 \). The mantle seems to be the major source of amino acids; this is supported by an increase in protein breakdown by the cathepsin A-, B-, H-, and L-like proteases. It is likely that the ubiquitin-proteasome system is activated, but this contention requires additional support. Although it is clear that mantle protein could support aerobic metabolism in mantle through the provision of amino acids, it is not known whether amino acids are released to fuel other tissues as well. We previously proposed that food deprivation in \textit{S. officinalis} results in gill remodeling (21), as observed in many fish species under adverse conditions (37). The new information on the rate of protein synthesis continues to support the proposition. There is no evidence on the basis of either enzyme activities or transcript levels that protein catabolism in gill of starved \textit{S. officinalis} is higher than that of fed animals. It may be that gill restructuring during food deprivation is associated with the maintenance of ionic balance, protection of water soluble plasma metabolites, or excretion ammonia.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


