

RESEARCH ARTICLE

Observations on the flowering and fruit developments in *Halophila stipulacea* (Hydrocharitaceae) in the Aegean Sea (Turkey)

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Abstract

The flowering and fruiting of *Halophila stipulacea* (Forsskål) Ascherson were followed on the Turkish coasts of the Aegean Sea, and both male and female flowers were recorded at the same location for the first time. Male and female flowers were found between mid-April and late September. In shallow coastal water, male flowers were fewer than female flowers, while in deep zones, female flowers were fewer than male flowers. The female flowers of *H. stipulacea* had three styles, but four styles of flowers were also found exceptionally. At the research stations, the plants and flowers showed a lower limit of 30 m depth and an upper limit of between 1 and 8 m. The upper limit of female plants was 4 m, and that of male plants was 6 m. Besides, flowering was recorded generally in very dense populations.

Keywords: Flower development, flowering, fruiting, pollen grain, pollination, seagrass

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Introduction

With the opening of the Suez Canal in 1869, *Halophila stipulacea*, which is assumed to have originated from the Indian Ocean (Aleem 1948; den Hartog

1970; Short *et al.* 2007), entered as a “Lessepsian immigrant” (Procaccini *et al.* 1999) from the Sinai coasts of the northern Red Sea to populate the eastern Mediterranean basin (Lipkin 1975; Lipkin and Silva 2002): in the north, the Aegean coasts of Greece and Turkey, along the North African coasts of Tunisia and Libya (Missaoui *et al.* 2003; Sghaier *et al.* 2011), and the coasts of Malta and Sicily, and from there into the western Mediterranean basin (Procaccini *et al.* 1999; Gambi *et al.* 2009).

In the eastern basin, despite many studies of its distribution in the Levant, Cyprus, Rhodes, and other countries on the Mediterranean coasts (Forti 1928; van der Velde and den Hartog 1992; Lipkin *et al.* 2003; Galil 2006; Gambi *et al.* 2009; Sghaier *et al.* 2011), little information has been available on the reproductive structure of this species (Procaccini *et al.* 1999; Xhulaj and Kashta 2007; Gambi *et al.* 2009), although the oldest records of flowering in the Mediterranean (female flowers and fruit only) were found in Fritsch (1895) for the island of Rhodes. In spite of the existence of several studies of its occurrence on the Greek coast of the western Aegean and the islands, especially Chios, there was no information on its flowering until 2012 (Tsirika and Haritonidis 2005; Katsanevakis and Tsimis 2009; Tsimis *et al.* 2010; Katsanevakis 2011). In August 2012 there was a report of mature fruit (Gerakaris and Tsiamis 2015), but no findings related to male flowers have been made.

The aim of the present study was to investigate the generative structure of the Lessepsian and dioecious marine flowering plant *Halophila stipulacea* in the Aegean Sea.

Materials and Methods

Plants were collected by scuba diving on the coasts of Kaş (Levantine Sea), Marmaris (South Aegean Sea), and Çeşme (Middle Aegean Sea) at depths of 2-30 m (Figure 1). Parameters such as depth, water temperature, plant cover and plant reproductive status were recorded. Samples were taken monthly between January and December 2014 from the two stations on the Çeşme coast (Eşek Island and Hacettepe Beach) to describe reproductive characteristics of *H. stipulacea*.

Underwater images were taken of the areas from where samples were taken and of the study materials using a camera (Olympus OM-D E-M5). Description and imaging of plant organs was performed using binocular light microscopes (Olympus SXZ16 and BX51).

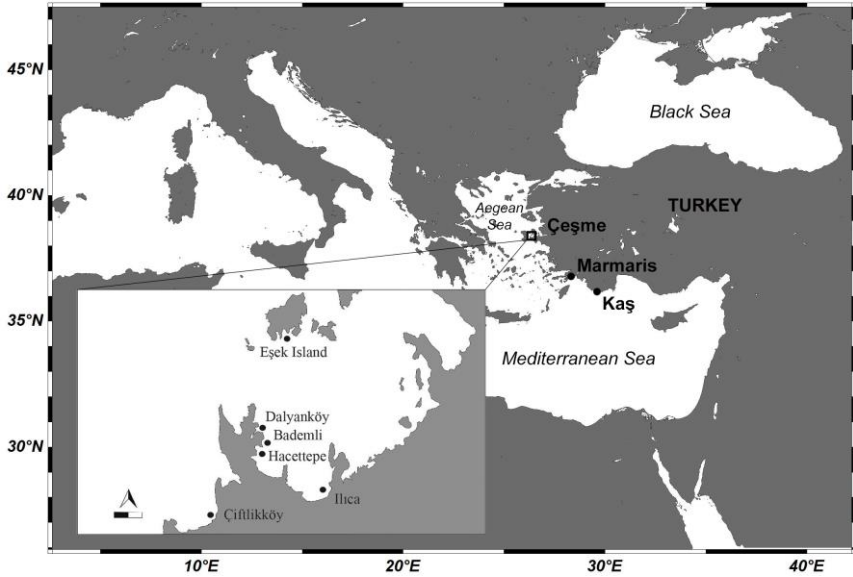


Figure 1. Sampling localities on the coasts of Turkey.

Results

Vegetative features

Plants dioecious, creeping, and with much-branched stolons forming extensive herbs (Figure 2A, B). Rhizomes; 1.5-2 mm in diameter. From each node emerges a shoot consisting of 1 root and 1(-2) leaves. Roots twisted or bent, 4-10 cm and densely hairy. Internodes 0.2-4 cm, 3-4 apical shoots and internodes very narrowed or 0.2 cm. The leaf sheath is obovate and hyaline, 0.8-1.5 cm long, 0.6-1 cm in width, 2 apical lobes separated obtusely along a dorsal line, surrounding the leaf and the internode towards the apical part of the shoot, with the sheath lost from old shoots (Figure 2C). Leaf-blade linear to oblong, green, glabrous, papillose or slightly hairy occasionally bullate, apex obtuse. Leaves; 1.7-8.2 cm in length, 0.2-1 cm wide, with up to 50 branched cross-veins connecting the intramarginal veins, which are parallel to the midvein (Figure 2D); base cuneate or gradually decurrent into the petiole; margin serrulate, especially in the apical region. Petiole; 0.3-1.5 cm long, leaves in pairs on 1-15 mm long shoots.

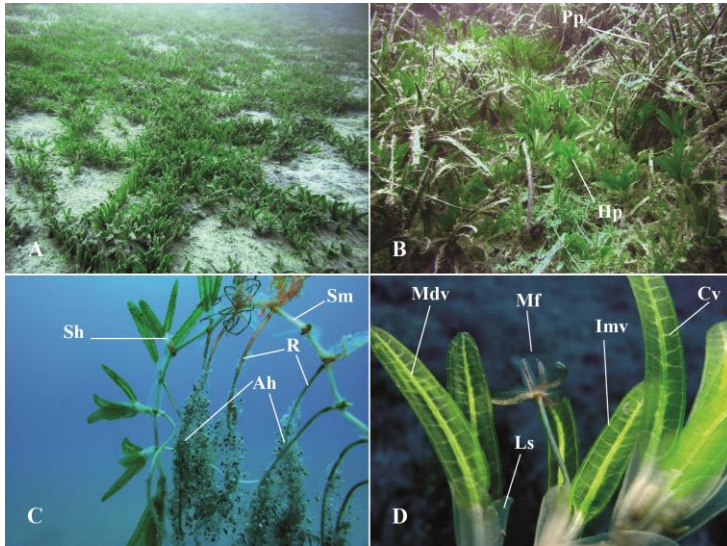


Figure 2. General vegetation and morphology of *Halophila stipulacea*
A: Hacettepe Beach. **B:** Eşek Island. *H. stipulacea* population (Hp), *Posidonia oceanica* population (Pp). **C:** The Vegetative Plant. Root (R), Absorbent hairs (Ah), Shoot (Sh), Stem (Sm), **D:** The staminate plant. Pair of leaves and midvein on leaf blade (Mdv), Male flower (Mf), Intramarginal vein (Imv), Cross vein (Cv), The Leaf-sheath (Ls).

A small number of species in the genus *Halophila* are monoecious: *H. decipiens* Ostenfeld, *H. beccarii* Ascherson, and *H. capricorni* Larcum, as well as *H. stipulacea*. However, the flower formation in the three monoecious *Halophila* species is different (Kuo and den Hartog 2006). Both male and female floral shoots of *H. decipiens* are produced at the same rhizome nodes (Kuo and den Hartog 2006). For the species without erect stems such as *H. ovalis*, *H. stipulacea*, and *H. decipiens*, flowers develop and mature progressively at each node along the rhizome branches. These results in the oldest fruits being furthest from the youngest shoot apex (Kuo and den Hartog 2006).

From mid-April to the first weeks of May female flowers of *H. stipulacea* were seen as pistils with long styles. Nevertheless, in the first weeks of May ovaries can be seen in which fertilisation has been completed (Figure 3). It indicates that mature male flowers must also exist at this time, but generally, fertilisation of female flowers occurs most from the middle of July to the end of the month, and the styles begin to fall away. From August to the end of September, mature fruit was more often seen, but young female flowers carrying styles can also be found at the apex of shoots (Figure 3A). The first shoot (1st) carried the oldest flower and newer flowers (2nd and 3rd) developed towards the apex (Figure 3B). Generally, few male flowers (2–3 on 15–20 segments) were found up to the end of September.

Mature flowers were generally seen in August and September. Nevertheless, flowers have been observed with tepals still unopened on Eşek Island in August. When the tepals open, the mature stamens split down their length and release their pollen in the form of chains of mucilage (Figure 3C), which are carried by the current and attach to the styles of female flowers. The nuclei inside the pollen grains extend from the hypanthium, which functions as a style, into the ovary, where fertilisation takes place. The styles fall away in three or four parts (Figure 3D, E), the seeds within the fruit begin to develop and the fruit matures and enlarges (Figure 3F).

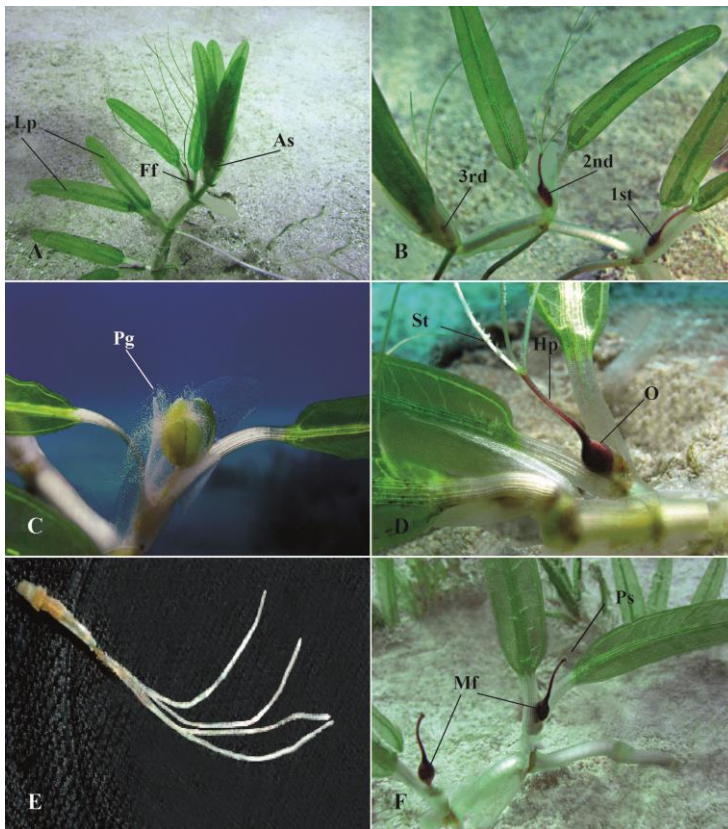


Figure 3. Morphology of *Halophila stipulacea*

A: Internodes of rhizomes and erect stems. Apical shoot (As), Female flower (Ff), Leaf pairs (Lp). **B:** Sequence of female flowers becoming younger towards the apical shoot.

C: Chain of pollen grains in mucilage (Pg). **D:** Mature female flower, Styles (St), hypanthium (Hp), ovary (O). **E:** The young female flower with four styles. **F:** Mature flowers with style fallen away after pollination (Mf) /fruit, papillate stigmata (Ps)

(Çeşme, 4-5 May 2013).

These results show that both male and female flowers are produced sequentially from mid-April to the end of September, and that fruit and seed development occurs.

Description of male flowers: In staminate plants, spathes or spathe bracts are 5–6 mm. Pedicels are 3–6 mm long. Tepals are 3–4 mm long (Figure 4A, B). Mature stamens are $2.0\text{--}3.5 \times 0.5\text{--}1.0$ mm in diameter (Figure 4C). As in all seagrasses, pollination is hydrophilic. Anthers split lengthwise and release pollen chains (Figures 4D and 5A). The mucilage of each pollen grain joins with each other and creates regular mucilage strings, protecting the pollen in this covering (Figure 5B, C). Pollen grains are elliptical to spheroid, 50×70 μm , spinulate; each pollen grain consists of an extine and an intine layer and the nuclei within it can be easily distinguished (Figure 5D).

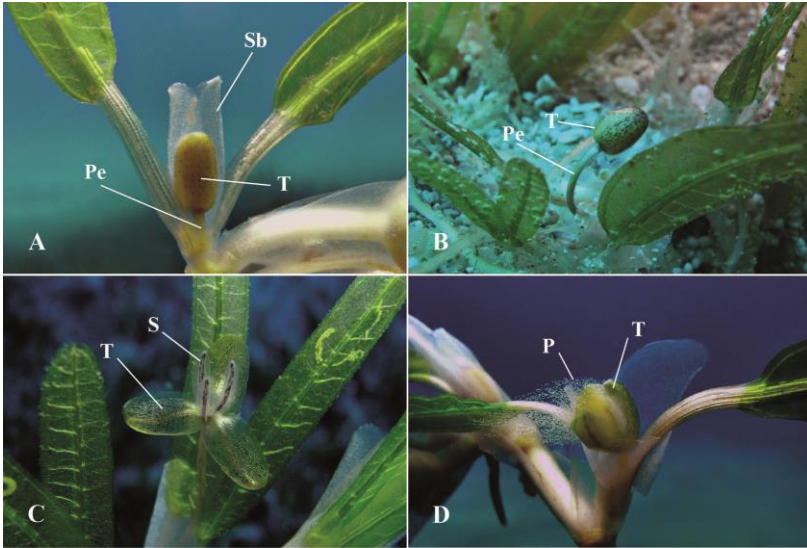


Figure 4. Morphology of male flowers of *Halophila stipulacea*
A-B: Pre-anthesis male flower with an extending pedicel (P) and pigmented tepals (T) enclosed by spathe bracts (Sb). **C:** Anthesis of the male flower and maturing stamens (S). **D:** Open to release pollen grains (P) in chains.

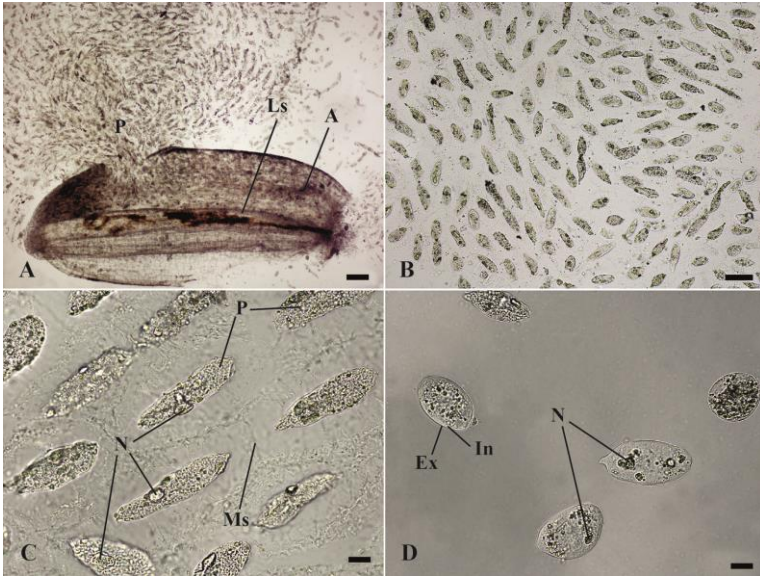


Figure 5. Ultrastructure of stamen and pollen grain of *Halophila stipulacea*

- A:** Open to release pollen grains in chains with lengthwise split (Ls) of anthers (A).
B: Chains of pollen in mucilage sheath. Scale=100 μm . **C:** Individual pollen grains in mucilage. Scale=20 μm . Mucilage sheath (Ms), Pollen grain (P), nucleus (n) **D:** Internal and external membranes in the pollen grain. Extine (Ex), Intine (In). Scale=20 μm .

Description of female flowers and fruits

In pistillate plants, spathes are vase-shaped, 1 cm long and 3–4 mm wide, ovate, acute, keeled, enclosing the female flower (Figure 6A, B). The ovary is ovoid to ellipsoid, yellow-green in colour, 1.5–3 mm long and 1–2 mm wide (Figure 6A). Mature fruit is coconut-like, violet in colour, 3–5 mm long and 2–3.5 mm wide, hypanthium is 2.5–3 times the length of the fruit, styles are filiform, 3 (–4) pairs and 3.5–4 cm in length (Figure 3D–F). However, it was found that in the southern Aegean (Marmaris), female flowers and fruits were smaller than the Çeşme samples (Figure 6B). After anthesis, the styles fall away, but the hypanthium remains. The ovary continues to develop with ovules 100–150 μm in size attached parietally to it (triple parietal placentae) (Figure 6C). The pericarp of the fruit is leathery and 30–100 ovoid or spherical seeds form after fertilisation. Sub-spherical seeds separate from the pericarp wall together with the funiculus in the form of a stalk. The seeds were measured as up to 700 μm long including the apical projection and 600 μm wide (Figure 6D). The distance between the two layers of the testa integument is 20–45 μm . The inner layer is formed from an amorphous-viscous material and envelopes the endosperm. The pre-embryo is nourished in this liquid and the first cotyledon primordium develops in the semicircular hypocotyl in the apex (Figure 6E).

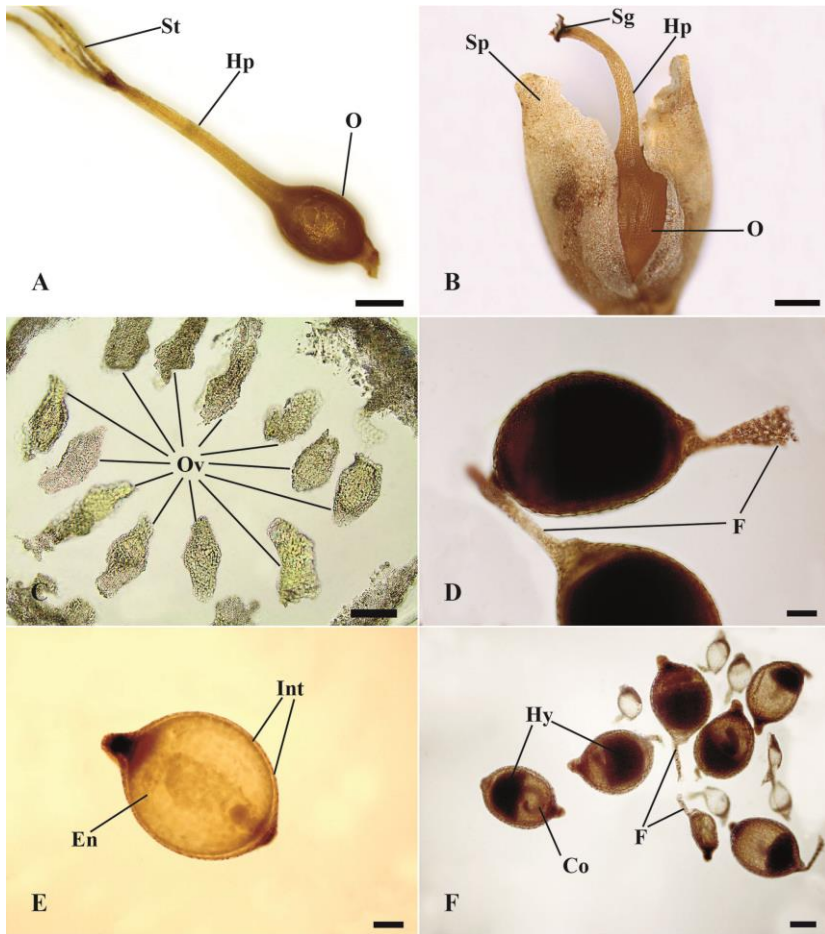


Figure 6. Morphology of female flower, fruit and seeds of *Halophila stipulacea*
A: Young pistil. ovary (O), hypanthium (Hp), styles (St); Scale=1 mm. **B:** Mature fruit (Marmaris, 28 August 2015), stigma (Sg), spathe (Sp); Scale=500 μ m. **C:** Stereobinocular microscope photographs of ovules. Scale=100 μ m. Ovule (Ov). **D:** Seeds with funiculus, funiculus (F); Scale=100 μ m. **E:** Embryo phase of seed. Scale=100 μ m. Integuments (Int), endosperm (En). **F:** Mature and young seeds. Scale=200 μ m. Funiculus (F), cotyledon (Co), and hypocotyl (Hy).

The most significant characteristic of seagrass embryos is the short life of their endosperms and their very large hypocotyl, and here it has been determined that the hypocotyl plays more of a role in storing nutrients than in germination (Kuo and den Hartog 2006). Of the parts that form the embryo, the cotyledon is helical, 1.5 mm in length, and semicircular (Figure 6F). After the fruit matures and after it is broken up naturally or mechanically, the seeds disperse in the water (Figure 7A, B). The fruit naturally opens by splitting along its length

(Figure 7C), and merger or longitudinal lines are found in mature seeds (Figure 7D). The seed testa has a leathery protective structure with the surface cells forming a hexagonal honeycomb pattern, and the intercellular areas of the surface cells frequently show a pore-like micro-structure (Figure 7E, F).

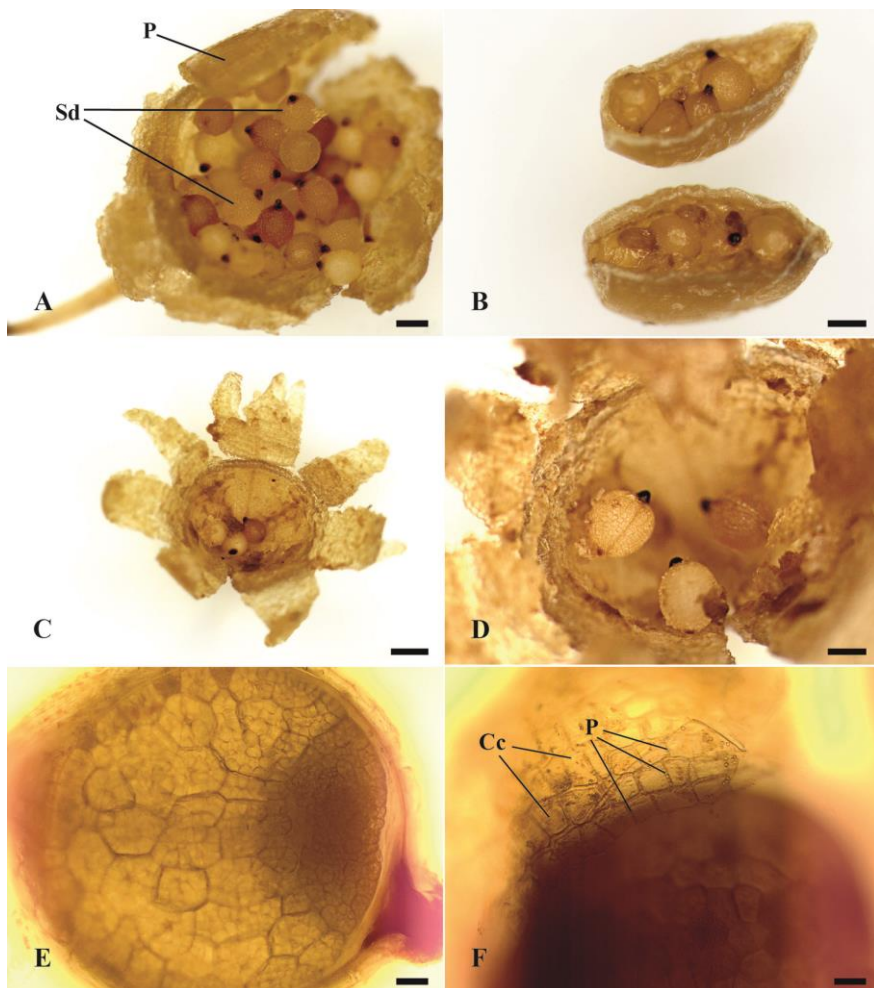


Figure 7. Morphology and ultrastructure of fruit and seeds of *Halophila stipulacea*
A: Stereo-binocular microscope photographs of mechanically broken fruit. Seeds (Sd) and pericarp (P), Scale=500 μm . **B:** Longitudinal section of fruit. Scale=200 μm .
C: Opening of naturally longitudinally split fruit. Scale=500 μm . **D:** Longitudinal merger line on seed (ML.) Scale=200 μm . **E:** Surface cells of testa and pores. Testa surface. Scale=50 μm . **F:** Cortex cells (Cc), pores (P). Scale=50 μm .

Discussion

Florescence in marine flowering plants is related to yearly varying ecological conditions. It can be seen from its spread and its flowering records on the coasts of Turkey that the increase in the generative organs of *H. stipulacea* in recent years may be linked to temperature. However, records of flower organs from the Mediterranean vary. In the western Mediterranean, male flowers have been found at shallow depths, if only rarely (Gambi *et al.* 2009). On the coasts of Turkey, male flowers are infrequently found at shallow depths, but female flowers are found in higher quantities (Table 1). At depth, female flowers are found infrequently, but male flowers are more often seen.

Exceptionally, a flower with four styles was reported among the samples examined (Figure 2E). This unusual appearance of four styli (instead of three) is also reported from other *Halophila* species such as *H. ovalis* (R. Brown) Hooker F. and *H. decipiens* Ostenfeld, and it is also common in populations of *H. decipiens* from Telugu in India (Bujang *et al.* 2006).

Short and Cambridge (1984) state that the reason for the paucity of male flowers of *H. stipulacea* was, as with *H. engelmanni*, the short life of these delicate flowers. We think that the small number of findings of male flowers is entirely related to close observation and low production. For this reason, some authors have reported different life styles according to the finding of flower organs. Thus, *H. beccari* has been reported as dioecious by Phillips and Menez (1988), and as monoecious by Zakaria *et al.* (1999) and Kuo and den Hartog (2001). The reporting of *H. beccari* as dioecious by Phillips and Menez (1988) may be because they were unable to find male and female flowers on the same plant at the same time, or because the male flowers had fallen earlier. Moreover, Phillips and Menez (1988) were unable to find male flowers on the species *H. hawaiiiana* Doty and Stone and *H. johnsonii* Eiseman. However, Kuo and den Hartog (2001) reported these species as dioecious (Table 2). Based on this information, it would be beneficial to monitor the florescence of marine flowering plants frequently, even at 15-day intervals, in a controlled way at both shallow and deep stations.

Table 1. Reproductive features and habitat characteristics of *Halophila stipulacea* populations on the coasts of Turkey

Region	Locality	Coordinates	Depth (m)	Salinity (‰)	Temperature (°C)	Substrate type	Reproductive status	Coverage (%)	Collected by
Levantine Sea	Kaş	36°08'49.74"N 29°41'42.27"E	20–23	39.2	27.9	sandy-rocky	male flowers	10–20	OKUDAN (July 2010)
Aegean Sea (South)	Muğla (Marmaris, Kızılkum)	36°48'21.91"N 28°16'23.17"E	6	37	27	sandy- muddy	male and female flowers	20–30	OKUDAN (August 2015)
Aegean Sea (South)	Muğla (Marmaris, Kızılkum)	36°48'21.91"N 28°16'23.17"E	6	37	25	sandy- muddy	male flowers	20–30	OKUDAN (September 2015)
Aegean Sea (Middle)	Çeşme (Çiftlikköy)	38°18'13.44"N 26°16'39.40"E	4–7	38.7	17	sandy	vegetative plants	20–30	DURAL (April 2012)
Aegean Sea (Middle)	Çeşme (Dalyanköy Cape)	38°21'16.74"N 26°19'29.27"E	23	38.9	24	sandy	vegetative plants	5–10	DURAL (July 1996)
Aegean Sea (Middle)	Çeşme (Bademli Cove)	38°21'01.07"N 26°19'22.53"E	4–13	38.8	23–24	sandy- muddy	male and female flowers	10–20	DURAL (June–September 2011–2014)
Aegean Sea (Middle)	Çeşme (Ilica Coast)	38°18'41.70"N 26°22'44.11"E	4	38.7	24–25	sandy	female flowers	20–30	AYSEL (June–September 2008–2014)
Aegean Sea (Middle)	Çeşme (Haceteppe Beach)	38°20'45.39"N 26°19'05.62"E	7;8;9;10	38.8	16–24	sandy	male and female flowers	10–20	DURAL April–September 2011–2014)
Aegean Sea (Middle)	Çeşme (Eşek Island)	38°25'16.48"N 26°19'51.77"E	4;5;6;9;12;1 3;20;26;27; 30	38.8	16–23	sandy	male and female flowers	10–20	DURAL (April–September 2011–2014)

Table 2. Comparison of various studies on the sexual status of *Halophila* species in the world (D: dioecious, M: monoecious)

Species	Womersley (1984)	Phillips and Menez (1988)	Kuo and den Hartog (2001)
<i>H. australis</i> Doty & Stone	D		D
<i>H. baillonii</i> Ascherson		D	D
<i>H. beccarii</i> Ascherson		D	M
<i>H. capricorni</i> Larcum *			M*
<i>H. decipiens</i> Ostenfeld	M	M	M
<i>H. engelmannii</i> Ascherson		D	D
<i>H. hawaiiiana</i> Doty & Stone		Male flowers unknown	D
<i>H. johnsonii</i> Eiseman		Male flowers unknown	D
<i>H. minor</i> (Zollinger) den Hartog		D	D
<i>H. ovalis</i> (R. Brown) Hooker f.	D	D	D
<i>H. ovata</i> Gaudichaud		D	D
<i>H. spinulosa</i> (R. Brown) Ascherson		D	D
<i>H. stipulacea</i> (Forsskål) Ascherson		D	D
<i>H. tricostata</i> Greenway		D	D

*Both male and female flowers are formed on the same floral shoot.

Short and Cambridge (1984) compared *H. engelmannii* with some other species such as *H. ovalis* (R. Brown) J. D. Hooker, including *H. stipulacea*, and mentioned the similarities of the structure of the male flowers. Although the temperature and salinity conditions of the Turkish coasts appear similar to those of the Indian River Lagoon, Florida, USA, according to our findings fewer male flowers were produced than female flowers and for this reason. Therefore male flowers were difficult to find, but they occurred in the same period as female flowers. In this way, mature fruits and fertile seeds are found both on the southern coasts and in the mid-Aegean from April to September. However, male flowering in the Indian River Lagoon was determined in the first week of May (Short and Cambridge 1984). On these coasts, mature fruits with style fallen away were found on 4 and 5 May. Also, the lack of findings of male flowers in shallow areas in the eastern Mediterranean over a long period may be related to an insufficient number of male flowers being produced and therefore not be found, or they may be produced at the same time but they are more delicate than the female flowers. Besides, they grow at greater depths because they are affected by high temperatures and intense radiation. It may also be related to shortcomings in studies and observation. The fact that *Halophila* species are more often found to be dioecious maybe because there are significant differences in the ecological requirements of the male and female flowers.

During our studies, both shallow and deep habitats on the Çeşme coast and in the bays of Eşek Island were carefully sampled almost meter by meter over one year. At all stations, small communities of male plants were found under the canopy of the large-leaved *Posidonia oceanica*. This was also observed in the same habitats on the southern coast, and it was noted that the male flowers, unlike the female flowers, did not grow in open areas but preferred deeper waters and shaded or protected habitats. Thus, while Gerakaris and Tsiamis (2015) found mature fruit at depths of 3–5 m in the same way on Chios, the nearest island to Çeşme, they did not record male flowers. Therefore, the possible occurrence of male flowers at depth can be understood from the occurrence of fertile fruit. In various studies conducted before 2009, only male flowers were found (Cancemi *et al.* 1994; Procaccini *et al.* 1999), but later Di Martino *et al.* (2000) found both male and female flowers. Gambi *et al.* (2009) again reported male flowers in a habitat with *P. oceanica* meadows at a depth of 2 m in the harbour of Palinuro in the province of Salerno, Italy in the central Tyrrhenian Sea, but did not find female flowers.

Conclusion

Two points stand out as the result of this study. The first is that in the western Mediterranean male flowers are more common than females, even in shallow waters, while in the eastern Mediterranean female flowers are found more commonly and densely at shallow depths. Male flowers are found very rarely at shallow depths, but in deeper waters, they are more common than female flowers. This may be because male flowers are more sensitive to temperature and light.

The second point is that *H. stipulacea* has exhibited a rapid vegetative spread from east to west and from south to north since its entry into the Mediterranean, and following this, temperature may stimulate the development of generative organs in generally dense populations and flowering (the occurrence of both male and female flowers, the preponderance of one over the other, and density) has been encountered increasingly frequently in recent years. It is thought that the increase in temperature in the waters of the Mediterranean and Aegean coasts has encouraged the rapid advance of populations towards the north as well as sexual reproduction. We believe therefore that careful monitoring and recording of flowering of *Halophila* and other seagrass species will be of great importance as indicators of temperature rise.

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***Halophila stipulacea* (Hydrocharitaceae) türünün Ege Denizi (Türkiye) kıyılarında dağılımı ve çiçeklenmesi üzerine gözlemler**

Öz

Bu araştırmada, Ege Denizi kıyılarında dağılım gösteren *Halophila stipulacea* (Forsskål) Ascherson türünün çiçeklenme ve meyve gelişiminin Nisan ayının ortalarından Eylül ayının sonlarına kadar devam ettiği, erkek ve dişi çiçeklerin aynı alanda dağılım gösterdiği tespit edilmiştir. Dişi çiçeklerin sığ, erkek çiçeklerin ise derin sularda sayıca fazla bulunduğu ve çiçeklenmenin dağılımının alt ve üst sınırının 4-30 m olduğu gözlenmiştir.

Anahtar kelimeler: Çiçek gelişimi, çiçeklenme, meyve gelişimi, tozlaşma, denizçayırı

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